Analysis of cyclin dependent kinases in *Leishmania*

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Declaration

I hereby declare that this thesis has been composed by myself, that the work of which it is a record has been done by myself except where assistance has been acknowledged, that it has not been submitted in any previous application for a higher degree and that all sources of information have been specifically acknowledged by means of references.

Some of the results contained in this thesis have been presented in a conference as follows:

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Summary

The results obtained from the experiments presented in this study aimed to further explore the role of cyclin dependent kinases and cyclins in the protozoan parasite *Leishmania major*. Cdks in kinetoplastids, CRKs, are the key regulators that allow cells to progress through different cell cycle phases and promote parasite proliferation during infection.

In chapter 3 of this study, the results presented showed that *L. major* CYCA is capable of activating CRK3 in an *in vitro* kinase assay using histone H1 as substrate. The CRK3/CYCA active complex was then used to analyse the effect of the phosphorylation at the CRK3 activation threonine using a kinase activating kinase (yeast CAK or Civ-1). Phosphorylated CRK3 activity was compared to non-phosphorylated CRK3 and it was found that the phosphorylation promotes a 5-fold increase in kinase activity of the complex. The accessory protein Cks1 was assayed *in vitro* with the active CRK3/CYCA complex and it was shown that Cks1 might have an inhibitory effect when histone H1 substrate is used. The IC_{50} for two different kinase inhibitors (Flavopiridol and Indirubin) was determined for the *in vitro* CRK3/CYCA complex and compared with the values found for the *in vivo* purified CRK3. Similar values were obtained suggesting that the *in vivo* complex is indeed represented by the recombinant complex.

In the following chapter 4, yeast Civ-1 purified from *E. coli,* was used to try to phosphorylate, in a similar manner, the activation of threonine/serine residues from other *L. major* CRKs. The kinases assessed were CRK1, CRK2, CRK4, CRK6 and CRK7. None of these were phosphorylated by Civ-1 suggesting that the only CRK under this type of regulation is CRK3. *L. major* CRK1-4 and CRK6-8 were tested in kinase assays by mixing under described conditions with *L. major* CYC9 and kinase activities towards three different substrates were assessed. *L. major* CYC9 was not able to activate the above kinases and the kinase subunit that interacts with this cyclin could not be identified.

In chapter 5, the *L. major* CYCA was used to elucidate the characteristics of this cyclin *in vivo*. A gene disruption strategy aimed to replace the two genomic alleles of this protein gene by homologous recombination. Plasmids were developed with flanking regions of this gene placed in association with two different drug resistance genes, one for each of the allele's disruption. These constructs were not able to produce the first allele knock out suggesting that not only this gene might be essential but the levels of expression may also be important. Tagging *L. major* CYCA was also attempted *in vivo* using two different strategies (i.e. two different tagging systems). The first tag employed was the TAP tag syste. Although drug resistant transfected cell lines were obtained, no tag detection could be observed by western blot using different tag-specific antibodies (α-protein-A and α-calmodulin antibodies). The second tag employed was HA, the 9-amino acid sequence YPYDVPDYA, derived from the human influenza hemagglutinin (HA) protein. Plasmids that contained C and N-terminal HA tagged *L. major* CYCA were used to transfect WT cells and cells extracts of resistant cell lines analysed by western blot. Both C and N-terminal HA tagged CYCA were detected by the α-HA antibody. Following the confirmation of the presence of the tagged CYCA in the cell extracts an affinity purification using an HA affinity matrix was attempted and the matrix binding material was used in *in vitro* kinase assays. The presence of kinase activity towards Histone H1 confirmed that CYCA was being succesfully immunoprecipitated in complex with a kinase partner. The identity of the co-eluted CRK could be confirmed using specific α-CRK3 antibody that detected CRK3 in the eluted material.

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Table of abbreviations

AREs	AU-rich elements
CAK	CDC activating kinase
Cdk	cyclin dependent kinase
Cks	Cdc kinase subunit
CRK	cdc2-related kinase
CRP	C-reactive protein
CYC	Cyclin
FT	Flow through
GIPL	glycosylinositol phospholipid
gp	glycoprotein
GPI	glycosylphosphatidylinositol
IFN-γ	interferon gamma
IL-1	interleukin-1
kDNA	kinetoplast DNA
L. aethiopica	Leishmania aethiopica
L. amazonensis	Leishmania amazonensis
L. braziliensis	Leishmania braziliensis
L. chagasi	Leishmania chagasi
L. donovani	Leishmania donovani
L. infantum	Leishmania infantum
L. major	Leishmania major
L. mexicana	Leishmania mexicana
L. venezuelensis	Leishmania venezuelensis
LPG	lipophosphoglycan
MAPKs	Mitotic associated protein kinases
MAT-1	ménage à trois-1.
MHC	histocompatibility complex
NO	nitric oxide
pRb	retinoblastoma protein
PSG	promastigote secretory gel
RINGO	rapid inducer of G2/M progression in oocites
RNAP	RNA polymerase
S. cerevisiae	Saccharomyces cerevisiae
SLRNA	spliced leader RNA
T. brucei	Trypanosoma brucei
T. cruzi	Trypanosoma cruzi
Th	T-helper
TNF-α	tumour necrosis factor alpha
UTR	untranslated region
V	Viannia
VSG	variant surface glycoprotein

Chapter 1

General Introduction

1.1 General introduction

Kinetoplastids are a remarkable group of protists and sophisticated eukaryotic parasites. They contain a range of ubiquitous free-living species, as well as pathogens of invertebrates, vertebrates and plants. They are unicellular organisms that have mastered unique solutions to the problems of being a eukaryotic cell. Kinetoplastid peculiarities include: complex and energy-consuming mitochondrial RNA editing; a unique mitochondrial DNA; the arrangement of genes into polycistronic units; *trans*-splicing of all mRNA transcripts; modifications of nucleotides; glycolysis in a separated organelle; use of diverse mechanisms to evade host immune response (Simpson et al., 2006). Kinetoplastids have a unique mitochondrial structure called the kinetoplast, hence the name Kinetoplastids (El Sayed et al., 2005).

Many important aspects of the biology of kinetoplastids are currently being addressed in many laboratories. These aspects, like flagellum biology, chromosome segregation, cell cycle and differentiation to list some, represents areas that are informed by similar studies in many other cell systems (Gull, 2001). Cell cycle and differentiation are among other aspects relatively unexplored. Little is known about the molecular mechanisms governing the cell cycle stages, its check points and its association with differentiation. Since parasitism with vicious consequences to the host will always depend upon cell proliferation, survival and differentiation the understanding of the cell cycle machinery can provide interesting new drug targets fighting diseases caused by kinetoplastid parasites.

At the present time, no entirely satisfactory treatment exists to intervene in almost all of the human and animal parasitic protozoan derived diseases. Prevention is also a problematic issue with vaccine development, which has proven inefficient. Current biomedical research has its focus on the search for newer intervention strategies, to control the public health impact of parasitic diseases. The dramatic advances of molecular and cellular biology in

recent times have provided opportunities for discovering and evaluating molecular targets for drug design, which now form a rational basis for the development of improved anti-parasitic therapy. Many cell processes including cell cycle, differentiation and hence proliferation depends on protein kinases. The importance of protein kinases in cell signaling and cell cycle control has led to detailed structural and functional studies in various eukaryotes, and hence to the synthesis of specific chemical inhibitors for managing disease (Doerig *et al.*, 2002;Scapin, 2006;Margutti and Laufer, 2007).

Protein phosphorylation has been documented in protozoan parasites for a number of years (Hermoso *et al.*, 1991;Doerig *et al.*, 2005). An increasing number of protein kinases of parasitic protozoa are being evaluated as drug targets, just as they had been in trials to treat a wide range of other diseases and syndromes, such as cancer, cardiovascular disease and Alzheimer's disease (Johnson, 2007). In recent years, pharmaceutical companies have invested heavily in the development of new compounds directed against specific protein kinase targets, and there are a wide range of protein kinase inhibitors that have entered clinical trials (Naula et al., 2005;Johnson, 2007).

In eukaryotic organisms, the cell cycle is regulated in part by the production and destruction of cyclins. When expressed, cyclins associate with specific kinases (cyclin dependent kinases) (Cdk) making an active pair that drives the cell cycle through its distinct phases. The Cdk family was found to be relatively large in trypanosomatids with 11 members in *Trypanosoma brucei* (*T. brucei)* and *Leishmania major* (*L. major)* and 10 in *Trypanosoma cruzi* (*T. cruzi)* (Naula *et al.*, 2005;Parsons et al., 2005). This is similar to what is observed in mammalians cells, where a large number of CDKs and cyclins are present, and different to unicellular yeast, which has only one CDK. The complexity observed in kinetoplastids may reflect the problem of dividing a highly polarized cell with an elaborate cytoskeleton and a single mitochondrion, along with an integral link between cell cycle control and life cycle differentiation. Despite the existence of a large number

cyclins and of CDK family members (named CRK for cdc2-related kinase), their role in the biology of these parasites is poorly understood.

T. brucei CRK3 was shown to form a complex with CYC6 mitotic cyclin and CYC2 (van Hellemond et al., 2000;Hammarton et al., 2003a). While CYC2 (named also cyclin E1) is essential for G1/S phase progression (Li and Wang, 2003b) CYC6 and CRK3 have been associated with the G2/M phase (Tu and Wang, 2004). A screen for possible interactions among the five CRKs (CRK1-CRK4, CRK6) and seven cyclins genes from *T. brucei* using yeast two-hybrid assays showed that CYC2 also interacts with CRK1 and CRK2 (Gourguechon et al., 2007). In *Leishmania donovani (L. donovani),* CRK3 was shown to interact with CYCA in the G1 phase (Banerjee et al., 2006). In *Leishmania mexicana* (*L. mexicana)* CRK3 was shown to be regulating G2/M phase transition (Hassan et al., 2001).

More detailed and precise functional studies, as well as information about the CRKs, cyclins, substrates and activators is urgently needed to establish if there are significant differences in cell cycle regulation between these parasites and its mammalian hosts, and hopefully, some of these differences will be exploitable in the future in order to develop new anti-parasitic drugs.

1.2 Epidemiology of kinetoplastid parasites

Kinetoplastid species are the aetiologic agents of a broad spectrum of tropical and subtropical diseases including the leishmaniases (*Leishmania* spp.), African sleeping sickness (*T. brucei*) and Chagas' disease (*T.cruzi*).

Trypanosoma brucei and its relatives are responsible for diseases in humans ("sleeping sickness") and livestock cattle in equatorial Africa ("nagana"). *T. brucei* undergoes complex cycles of differentiation and multiplication in two very different hosts, the tsetse vector and the vertebrate host. Two forms of human sleeping sickness are recognised, a

chronic form found in West and Central Africa, caused by *Trypanosoma gambiense*, and a more acute disease found in East Africa caused by *Trypanosoma rhodesiense*. Both these trypanosomes have similar morphology and are transmitted by tsetse flies, characteristics shared with *T. brucei* (Gibson, 2001).

T. cruzi is responsible for Chagas' disease. It primarily affects rural South America but also constitutes a potential hazard in Mexico and the United States, primarily through blood and organ donations. Chagas disease is the clinical condition triggered by infection with the protozoan *T.cruzi*. The infection is transmitted by triatomine insects while blood feeding on a human host (Teixeira et al., 2006). The disease was described in the pioneering work of Dr Carlos Chagas, a Brazilian physician who worked at the Oswaldo Cruz Institute, Rio de Janeiro, in 1909 (Chagas, 1909).

Leishmania parasites are named after W.B. Leishman, a Scot, who developed one of the earliest stains for *Leishmania* in 1901. Today, 30 species are known and approximately 20 are pathogenic for humans (Murray et al., 2005a). Widespread in 22 countries in the New World and in 66 nations in the Old World and occurring in several forms, the disease is generally recognized for its cutaneous form which causes non-fatal, disfiguring lesions, although epidemics of the potentially fatal visceral form cause thousands of deaths. The species that infect humans cause a different spectrum of symptoms. These range from simple, self-healing skin ulcers (e.g. due to infection with *L. major*), to severe, life-threatening disease (e.g. visceral leishmaniasis caused by *L. donovani*). In between, mucocutaneous leishmaniasis, due to *Leishmania braziliensis* (*L. braziliensis)* infection, begins with skin ulcers which spread, causing dreadful and massive tissue destruction, especially of the nose and mouth. Post-kala-azar dermal leishmaniasis is a complication of visceral leishmaniasis observed mainly in Sudan and India where it follows treated visceral leishmaniasis in sometimes 50%. Diffuse cutaneous leishmaniasis, although rare, is

associated with the absence of specific cell-mediated immunity to *Leishmania*. This disease shares features with visceral leishmaniasis (WHO, http://www.who.int).

1.2.1 General biology of the genus Leishmania

Leishmania is transmitted by the bite of certain species of sand fly, including flies in the genus *Lutzomyia* in the New World and *Phlebotomus* in the Old World. Human infection is caused by about 20 of 30 species that infect mammals. These include the *L. donovani* complex with three species, which are *L. donovani, Leishmania infantum (L. infantum)*, and *Leishmania chagasi* (*L. chagasi*); the *L. mexicana* complex with 3 main species, which are *L. mexicana, Leishmania amazonensis (L. amazonensis*) and *Leishmania venezuelensis* (*L. venezuelensis*); *L. tropica; L. major; Leishmania aethiopica (L. aethiopica)*; and the subgenus *Viannia* with four main species, which are *L. (V.) braziliensis, L. (V.) guyanensis, L. (V.) panamensis*, and *L. (V.) peruviana*). The different species are morphologically indistinguishable, and can only be differentiated by isoenzyme analysis, DNA sequence analysis and monoclonal antibodies. The pathogenic profiles of the diseases provoked by the species inside these groups are more distinguishable. *L. major* and the *L. mexicana* complex include subspecies that cause cutaneous leishmaniasis and diffuse cutaneous leishmaniasis. *L. donovani, L. infantum*, and *L. chagasi* from the *L. donovani* complex cause visceral leishmaniasis. Mucocutaneous leishmaniasis is caused by *L. (V) braziliensis*.

Leishmaniases are globally widespread diseases. The life-cycle starts when a parasitized female sandfly takes a blood meal from a human host (Figure 1.1). As the sandfly feeds, promastigote forms of the leishmanial parasite enter the human host via the proboscis. Within the human host, the promastigote forms of the parasite are ingested by macrophage where they metamorphose into amastigote forms and reproduce by binary fission. After amastigote multiplication and rupture of the macrophage, the amastigotes invade

neighbouring macrophages. The life cycle is complete when a phlebotomine female is infected while feeding on the blood of an infected host. The amastigotes released in the sand fly's intestine change into procyclic promastigotes before migrating towards the pharynx and the proboscis (Banuls et al., 2007).

Within the intermediate host, *Leishmania* develops as promastigote forms, elongated motile extracellular stages possessing a prominent free flagellum. A variety of different promastigote forms have been distinguished on morphological grounds (Banuls *et al.*, 2007). At least five distinct *Leishmania* developmental *in vivo* forms can be detected: procyclic promastigotes, nectomonad promastigotes, haptomonad promastigotes, paramastigotes and metacyclic promastigotes (Bates, 1994). The first developmental event after the blood meal is the passage from amastigote to procyclic promastigote. Proliferation of procyclic promastigotes occurs in the peritrophic membrane of the insect tract. After three days in the insect, differentiated nectomonad promastigotes make their move to the posterior midgut spreading the infection to the anterior midgut. Haptomonad promastigotes are found attached in the stomodeal valve. Five days after infection, highly motile, human infective metacyclic promastigotes are observed in the lumen of midgut and foregut (Kamhawi, 2006). Human infective insect forms invest considerable resources in the biosynthesis of promastigote secretory gel (PSG), which is a key factor in promoting transmission (Bates, 2006). Also, just before infection, and still inside the insect, occurs the metacyclogenesis: the differentiation of non-dividing metacyclic promastigotes, the actual human infective form. As different kinds of promastigote occur in the sand fly phase of the life cycle, it is important to note that synthesis of PSG only occurs at stages that are human infective. Metacyclic promastigotes are pre-adapted for survival in the mammalian host, they are complement resistant, express stage-specific genes and are biochemically part-way to becoming amastigotes.

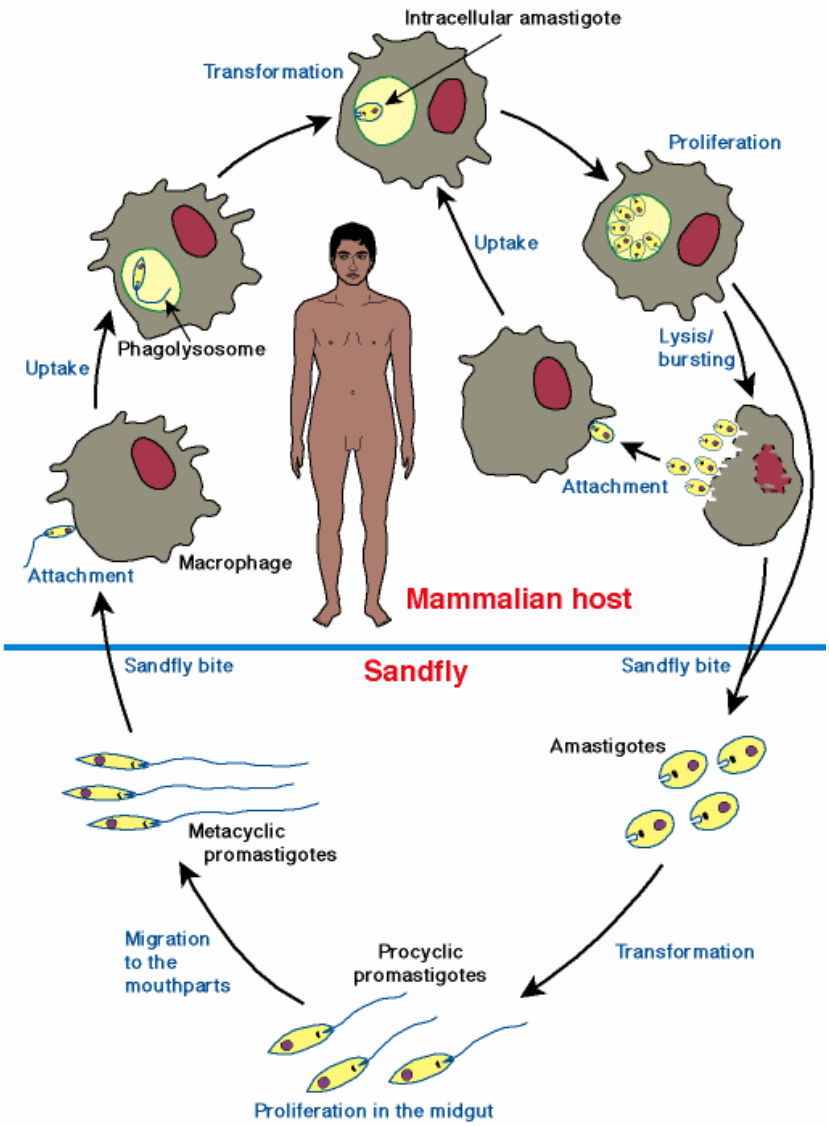


Figure 1.1 The *Leishmania* life cycle. As *Leishmania* parasites cycle through the mammalian host and the sand fly they encounter a variety of different environments to which they have to adapt and survive. They alternate between dividing-non-dividing forms, intracellular-extracellular forms, and nonflagellated-flagellated forms.

1.2.1.1 Mechanisms of escape

Leishmania are extremely successful parasites and natural infections are found in many different mammals, including dogs, rodents, marsupials, humans and other primates (Murray *et al.*, 2005a). All these vertebrates are considered as potential reservoirs of the disease. Inside the vertebrate host, the parasite evolves into an amastigote form. Amastigotes are ovoid (2.5–5 μm diameter), nonmotile intracellular stages that are formed by differentiation after being internalized by macrophages. They do not have a free flagellum and are located in the parasitophorous vacuoles of the host's macrophages. *Leishmania* is capable of successfully parasitize the macrophages that are the mammalian cells responsible for their killing.

Leishmania developed a range of sophisticated mechanisms to subvert normal macrophage function. These include preventing the activation of deadly antimicrobial agents such as nitric oxide (NO) and also inhibition of many of the cytokine-inducible macrophage functions necessary for the development of an effective immune response. Contact between the parasite and the macrophage prevents the macrophage from responding to subsequent exposure to interferon gamma (IFN-γ), interleukin-1 (IL-1) and tumour necrosis factor alpha (TNF-α). These factors are also directly inhibited by the parasite. This enables the parasite to evade the innate immune response and to divide within the phagolysosome of the infected macrophage, from where it can spread and propagate the disease within the host (Olivier et al., 2005).

One important surface molecule is the glycoprotein gp63 (promastigote surface protease). This is a zinc-dependent metalloprotease with a wide range of substrates, including casein, gelatin, albumin, hemoglobin, and fibrinogen (McMaster *et al.*, 1994). While around 10-fold less abundant than lipophosphoglycan (LPG), gp63 is still found throughout the promastigote surface (Pimenta et al., 1991;McConville et al., 1995). However, its shorter

length means that it is essentially buried under LPG. Like LPG, gp63 is down-regulated in the amastigote form (Schneider *et al.*, 1992). This reduced expression may be counteracted by the absence of LPG on the amastigote surface, meaning that gp63 is no longer masked and may therefore play an important role in amastigote survival and modulation of the host response (Yao *et al.*, 2003). In *L. major* and *L. amazonensis* the gp63 membrane protease converts complement protein C3b into C3bi promoting opsonisation and internalization by macrophages. *L. major* metacyclic promastigotes LPG are longer that LPG in procyclic metacyclic and shown to prevent complement system cellular lysis by avoiding the binding of C5b-C9. LPG can also interact with C-reactive protein (CRP), an early inflammatory product, and thus triggers phagocytosis via the CRP receptor. The gp63 surface molecule has optimal activity at the acidic pH found in phagolysosomes, supporting the suggestion that it targets lysosomal enzymes. However, its role is questionable, as parasites with mutations in the six gp63 genes are still capable of survival, differentiation, and replication within macrophages. One study of 245 macrophage genes showed that 37% were repressed at least twofold following *in vitro* infection with amastigotes (Buates and Matlashewski, 2001) which could explain how parasites can survive without gp63 inside the macrophage.

1.2.1.2 Immune response

Since many individuals remain asymptomatic, it is obvious that the natural immune response of humans can eliminate or control the parasites to a certain extent. While neutrophils are the first cells to arrive at the site of infection macrophages are the cells to be parasitized by *Leishmania*. Neutrophils interact with macrophages and regulate *L. major* infection by activation leshmanicidal activity in macrophages (Ribeiro-Gomes *et al.*, 2004). Macrophages are key cells in the host immune defense (Basu and Ray, 2005), as well as dendritic cells, that present the parasite antigens to T cell receptors, via the major histocompatibility complex (MHC) molecules. Antibody production or cellular cytolytic immune response will be unleashed depending on the cytokine context, on the *Leishmania*

peptides presented and which MHC class is being used (Banuls *et al.*, 2007). However, *Leishmania* parasites have evolved mechanisms to evade or interfere with antigen presentation processes, making it possible to partially resist the T cell-mediated immune responses (Antoine *et al.*, 2004). These escape strategies appear complex and various since, in humans, different patterns of immunological response are observed according to the clinical manifestation and exposure to different *Leishmania* species. Different T cell responses are observed among the different cutaneous forms of leishmaniasis: an absence of a T-helper-1 (Th1) response (rather than presence of Th2) in diffuse cutaneous leishmaniasis; a Th1 response with self-healing lesions (Kemp *et al.*, 1994;Ajdary *et al.*, 2000); and a mixed Th1/Th2 response with high IFN-γ levels with mucocutaneous leishmaniasis (Melby *et al.*, 1994;Louzir *et al.*, 1998;Bacellar *et al.*, 2002). In visceral leishmaniasis, a mixed Th1/Th2 response is observed with production of IFN-γ along with IL-10 (Ghalib *et al.*, 1993;Kenney *et al.*, 1998). However, individuals with asymptomatic or subclinical infections of visceralizing species of *Leishmania* show peripheral blood mononuclear cell proliferation and production of IL-2, IFN-γ and IL-12; in cured patients, both Th1 and Th2 clones producing IFN-γ and IL-4 have been isolated (Kemp *et al.*, 1993).

1.2.1.3 Membrane

Leishmania parasites can be distinguished by their surface molecule composition. Procyclic promastigotes are covered by a 7-nm-thick glycocalyx. The glycocalyx of metacyclic promastigotes is 17 nm. It is almost completely absent from amastigotes (Pimenta *et al.*, 1991). This surface comprises glycoproteins and other glycosylated groups, which are anchored to the surface membrane by a distinctive glycosylphosphatidylinositol (GPI) linkage (Ferguson, 1997). The dominant surface molecule of promastigotes is LPG. The structure of LPG varies between *Leishmania* species, but it is composed principally of repetitive units consisting of a disaccharide and a

phosphate, linked to the membrane by a GPI anchor. *Leishmania* species differ markedly by the presence of glycan side chains, as well as by their composition and positioning on the LPG core structure. LPG of *L. major*, for example, is highly branched, whereas that of *L. donovani* is not (McConville *et al.*, 1995). Furthermore, the structure of LPG differs between procyclic and metacyclic promastigotes, being significantly longer in the latter, and is almost completely absent from amastigotes (Naderer *et al.*, 2004).

The most abundant promastigote surface molecule is glycosylinositol phospholipid (GIPL), a class of GPI-linked glycolipids. These molecules are 10 times more abundant than LPG, but their small size keeps them close to the parasite membrane, so it is unclear what role they play in interaction with the host. Unlike LPG, which is continually shed, GIPL has a long half-life and so is believed to play a protective role at the promastigote surface (Proudfoot *et al.*, 1995).

1.2.1.4 Disease diagnostic

Since morphologic differentiation of *Leishmania* species is not possible, a variety of diagnostic tests using biochemical, immunologic, or molecular tools were developed. Most diagnostic tests for *Leishmania* are genus specific but not species specific (Schwartz et al., 2006a). Systemic treatment is recommended for *L. viannia* species but is not required for most cases of other cutaneous leishmaniasis. Necessary or unnecessary systemic therapy may be harmful, since it can be associated with toxic adverse effects (Schwartz et al., 2006b). For that reason, it is important to have diagnostic tests that can distinguish between *L. viannia* and other leishmanias. Several PCR-based assays for species differentiation were developed. A simple method was described using multiplex PCR that allows simultaneous detection of the *Leishmania* genus and identification of the *L. braziliensis* complex (Belli *et al.*, 1998). This was achieved using kinetoplast DNA (kDNA) minicircles but a number of other methods using as the rRNA gene (van Eys *et al.*, 1992),

repetitive sequences (Piarroux *et al.*, 1995), the gp63 gene locus (Victoir *et al.*, 1998), microsatellite DNA (Russell *et al.*, 1999), internal transcribed spacer regions (Cupolillo *et al.*, 1995;Eisenberger and Jaffe, 1999) and the tubulin gene (Luis *et al.*, 1998) have also been described.

1.2.1.5 Disease treatment

At the present moment, treatment of cutaneous leishmaniasis aims to accelerate cure, reduce scarring, and to attempt to prevent dissemination like mucosal disease or relapse. Resilient and older than 6 months lesions are generally treated. Pentavalent antimony, paromomycin, miltefosine, pentamidine are some drugs used against the parasites. The other strategies are immunotherapeutic interventions.

Immunotherapeutic interventions have been used as a form of treatment since endogenous host mechanisms can lead to tissue damage of the host itself. Prevention against these host mechanisms is called immunointervention. Healing of diffuse cutaneous and mucosal leishmaniasis was achieved using injections of attenuated promastigotes (Convit *et al.*, 2004). Other approaches have also included use of activating cytokines, inhibition of TNF-induced inflammation and and topical immunomodulators (Murray et al., 2005c). In one study drug-refractory patients were successfully treated with a combination of Leishmania recombinant antigens (Badaro et al., 2001)

Pentavalent antimony is available in branded (sodium stibogluconate [Pentostam], meglumine antimoniate [Glucantime], and generic form. For cutaneous leishmaniasis antimony is administrated parenteral and intralesional. However, up to 20 daily injections of a moderately toxic drug causes as much morbity to patients as the disease itself (Murray *et al.*, 2005a). Carefully tested, well tolerated, inexpensive oral agents are urgently needed. Furthermore, there is no consensus on optimum treatment in cutaneous leishmaniasis in general, alternatives to systemic antimony are under active investigation (Murray et al.,

2005b). The first oral anti-leishmanial drug, Miltefosine (hexadecylphosphocholine), was an oral anticancer agent that has shown to be effective against *Leishmania* (Croft et al., 1987;Kuhlencord et al., 1992). Although it is an effective oral drug its teratogenicity makes Miltefosine still a drug of limited (Olliaro et al., 2005).

1.2.2 General biology of the genus Trypanosoma

Two more important species that causes disease in human and animals inside the genus *Trypanosoma* are the American trypanosome *T. cruzi* and the African trypanosome *T. brucei*. *T. cruzi* enters the human body though broken skin of the damage caused by the bite and bloodfeeding of bugs from the sub-family *Triatominae*. They emerge at night to bite and suck blood. While feeding these bugs dispose their faeces containing parasites which can enter the wound left after the bloodmeal, usually when it is scratched or rubbed (http://www.who.int/). *T. brucei* enters the bloodstream via the bite of bloodfeeding tsetse flies (*Glossina spp*). Male and female tsetse flies transfer the parasites from human to human. Cattle and other wild mammals act as reservoir hosts of the parasites. Tsetse flies can acquire parasites by feeding on these animals, or on an infected person. Inside the human host, *T. brucei* multiply and invade most tissues.

The natural immunity of humans to the cattle pathogen *Trypanosoma brucei brucei*, but not to the human pathogens *T. b. gambiense* and *T. b. rhodesiense*, is a result of the selective killing of *T. b. brucei* by normal human serum (Rickman and Robson, 1970). Normal human serum contains apolipoprotein L-I, which lyses African trypanosomes (Vanhamme *et al.*, 2003) except resistant forms such as *T. brucei rhodesiense*. *T. b. rhodesiense* expresses the apoL-I-neutralizing serum resistance-associated protein, endowing this parasite with the ability to infect humans and cause trypanosomiasis (Baral *et al.*, 2006). African trypanosomes undergo a complex life cycle when they move from the bloodstream of their mammalian host to the blood-feeding insect vector, the tsetse fly

(*Glossina* spp.). They encounter many different environments during their life cycle and respond to these by significant morphological and metabolic changes, including adaptation of their energy metabolism (van Hellemond et al., 2005) But the most remarkable adaptation to survival developed by these parasites is the constant change in their surface proteins. Trypanosomes are coated with a variant surface glycoprotein (VSG) (McCulloch, 2004) that is so densely packed that it physically protects underlying proteins from effectors of the host immune system (Marcello et al., 2007). When antibodies are produced to a specific VSG that is expressed by the population the parasite then switches to express a distinct VSG which, if antigenically novel, allows clonal proliferation of the switched cells, generating a new parasitaemia peak. Each trypanosome expresses only one VSG gene but has the potential to switch to any of probably hundreds of others (Berriman et al., 2005).

1.3 Genomic organization of kinetoplastid parasites

Trypanosomatids have a unique mitochondrion containing a kinetoplast (Lukes et al., 2005). This organelle has a uniquely structured DNA content called kinetoplast DNA and, as one of the largest existing organellar genomes, is assembled into two classes of molecules: dozens of maxicircles and thousands of minicircles. Minicircles are circular but non-supercoiled molecules that are typically 1 Kb in size and linked together (catenated) into a network that resembles chain mail armour. Maxicircles encode most of the mitochondrial genetic information but many transcripts are extensively edited by the insertions and/or deletions of uridines: a process controlled by numerous minicircle-encoded guide RNAs (Simpson et al., 2003).

Old World *Leishmania* (*L. donovani* and *L. major* groups) have 36 chromosome pairs (0.28 to 2.8 Mb), whereas New World species have 34 or 35, with chromosomes 8+29 and 20+36 fused in the *L. mexicana* group and 20+34 in the *L. braziliensis* group (Ivens *at al.*,

2005). Between *L. major, T. brucei* and *T. cruzi* a large-scale gene synteny over 200–500 million years was found (El Sayed *et al.*, 2005). However, the organization of the chromosomes of *Leishmania* differ from those of the trypanosome species in not having extended subtelomeric regions containing species-specific genes (Peacock et al., 2007b) .

The genomes of *L. infantum, L. brasiliensis* and *L. major* have around 200 genes or pseudogenes differences. There are also around 78 genes that are species specific (Peacock et al., 2007a) . The genomes of *L. infantum* and *L. brasiliensis* show significant differences to the genome of *L. major*. It was suggested that there are too few species specific genes. However, these genes are suggested to be important in pathogenesis. Furthermore, the main source of variations may be gene copy number and the parasite genome plays only a small part in determining the clinical aspects of the disease (Peacock et al., 2007c).

T. brucei have a 26-megabase genome contains 9068 predicted genes, including ~900 pseudogenes and ~1700 *T. brucei*–specific genes. Large subtelomeric arrays contain an archive of 806 VSG genes used by the parasite to evade the mammalian immune system (Berriman *et al.*, 2005;Barry et al., 2005).

1.3.1 Regulation of gene expression

It is generally accepted that the genome of kinetoplastids is organized in long, polycistronic transcription units, with batteries of genes oriented in the same direction (Gibson *et al.*, 1988;Tschudi and Ullu, 1988;Myler *et al.*, 1999). These genes are usually separated by only a few hundred base pairs and, with a few exceptions, they do not contain introns. The long polycistronic units seem to be permanently transcribed in proliferative stages of the parasite (Martinez-Calvillo *et al.*, 2003). Cellular differentiation is controlled primarily at the level of individual mRNA maturation and stability (Clayton, 2002). The transcription units of the two major stage-specific antigens, surface glycoproteins like the

VSG in *T. brucei* and procyclin of the procyclic form, are subject to an additional layer of control including RNA elongation and processing (Pays, 2005).

1.3.1.1 Transcriptional

In eukaryotic cells there are three distinct classes of nuclear RNA polymerase: RNAP I, II and III. Each class of polymerase is responsible for the synthesis of a different kind of RNA: RNAP I is involved in the production of 18S, 5.8S and 28S rRNAs; RNAP II participates in the generation of mRNAs and most of the small nuclear RNAs; while RNAP III synthesizes small essential RNAs, such as tRNAs, 5S rRNA and some snRNAs (Martinez-Calvillo et al., 2007) Transcription in the kinetoplastid protozoa shows substantial variation from other eukaryotic gene expression systems, including polycistronic transcription, few RNA polymerase II promoters, no differential transcription initiation factors for most protein-coding genes, transcription of some protein-coding genes by RNA polymerase I, an exclusive subnuclear location for *VSG* transcription, the dependence of small nuclear RNA gene transcription on an upstream tRNA gene, and the synthesis of mitochondrial tRNAs in the nucleus (Campbell et al., 2003;Martinez-Calvillo *et al.*, 2007). Promoters for RNA polymerase I (Pol I) have been extensively characterized in trypanosomatids (Rudenko *et al.*, 1995;Yan *et al.*, 1999), as have some Pol III promoters (Ben Shlomo *et al.*, 1997). In trypanosomatids, the lack of transcriptional regulation suggests the presence of distinct posttranscriptional mechanisms to control differential gene expression. It was demonstrated that mRNA transcripts of four genes accumulate at the beginning of S-phase, and degrade rapidly after DNA replication is completed. All the genes showing this post-transcriptional cell cycle-dependent regulation encode for proteins that participate in DNA metabolism (Zick et al., 2005;Banerjee *et al.*, 2006). In *Leishmania* the stability of S-phase specific mRNAs in these parasites is determined primarily by the presence of the octanucleotide sequence (C/A)AUAGAA(G/A) in the untranslated regions (UTRs) of the transcripts (Zick *et al.*, 2005). The promoter for the spliced leader RNA

genes recruits RNA polymerase II (Das and Bellofatto, 2003), whereas the three other known promoters recruit RNA pdymerase I, including the protein coding genes and promoters of the rDNA (Pays, 2005). Another evidence for RNA pol II transcription shows that this enzyme acts on the strand-switch region bidirectionally at a few sites along the chromosome and ends at the tRNA region (Martinez-Calvillo *et al.*, 2004).

1.3.1.2 Post-transcriptional

The general organization of genes in polycistronic units means that all mRNAs must be *trans*-spliced to be capped and that post-transcriptional regulation of the mRNA level is important for changes of gene expression (Campbell *et al.*, 2000). This regulation occurs through sequence elements in intergenic UTRs that determine stage-specific mRNA abundance (Drozdz and Clayton, 1999). In addition to that the spliced leader RNA (SLRNA) that provides a 5' cap to the polycistronically transcribed mRNA and has been implicated in mRNA translation (Zeiner *et al.*, 2003). Regulation of gene expression seems to happens mainly at the post-transcriptional level (Clayton and Shapira, 2007).

Post-transcriptional regulation of gene expression in kinetoplastids may be explained when the cell experiences a major stress that probably precludes the activation of energy-dependent mechanisms. Just changing the choice between pre-existing RNAs would be more efficient than switching promoter activity through the recruitment of specific silencers and/or activators as occurs in most eukaryotes. However, this requires the permanent degradation of an important fraction of the transcriptome and costs energy (Brems *et al.*, 2005). The components of a RNA turnover machinery has being partially described as the *T. brucei* exosome (Estevez *et al.*, 2001). Posttranscriptional processing and degradation of RNAs are performed by enzymes and ribonudeoprotein complexes regulated by *trans*-acting factors that bind the RNAs.

Recently, strong influence of the pre-ATG triplet on the level of protein expression over a 20-fold range was detected in *L. tarentolae* (Lukes et al., 2006). This study concluded that a conserved mechanism of translation initiation site selection exists in kinetoplastids, which is strongly influenced not only by the pre-ATG sequences but also by the coding region of the gene (Lukes *et al.*, 2006). Because stable and structural RNAs must be processed, modified, and assembled into ribonucleoprotein complexes posttranscriptional processing and degradation of RNAs are affected by enzymes and ribonudeoprotein complexes and regulated by *trans*-acting factors that bind the RNAs. The mRNAs from Kinetoplastids contain AU-rich elements (AREs) in their 3′ UTRs that seems to be important for mRNA metabolism like in mammalian cells (De Gaudenzi *et al.*, 2005).

However, the most remarkable aspect of trypanosomatid RNA metabolism is the editing of kinetoplast mRNA transcripts through the addition and deletion of uridine residues (Simpson *et al.*, 2003). This trypanosome-specific processes and organization evolved by the acquisition of a large number of foreign genes, which entered a trypanosomatid ancestor through lateral gene transfer. Many different organisms like viruses and bacteria, such as cyanobacterial endosymbionts and non-phototrophic bacteria were probable donors (Opperdoes and Michels, 2007).

1.4 The eukaryotic cell cycle

Many of the core components involved in the cell cycle are conserved across eukaryotes, both functionally and structurally (de Lichtenberg et al., 2007). The control is exerted at different levels like transcriptional regulation, phosphorylation, subcellular translocation and targeted degradation (de Lichtenberg *et al.*, 2007). The cell cycle and progression through its phases (G1, S, G2 and M) is regulated by several means including microRNAs, which target transcripts encoding proteins directly or indirectly involved in cell cycle progression and cellular proliferation (Carleton *et al.*, 2007). Also by iron (Fe) that regulate

and control molecules like p53, p27 (Kip1), cyclin D1 and cdk2 (Yu *et al.*, 2007). However, genome-scale data showed poor conservation of both the transcriptional and the post-translational regulation of individual genes and proteins involved in the cell cycle (de Lichtenberg *et al.*, 2007).

The cell cycle and progression through its phases is regulated by the production and destruction of cell cycle specific proteins called cyclins (Standart *et al.*, 1987). Different classes of cyclins are synthesized in each cell cycle stage where they associate with CDKs activating the kinase (Pines and Hunter, 1991). The kinase activity is believed to be the driving force behind the cell cycle progression. CDKs are relatively small proteins (~34 kDa) containing little more than a protein kinase catalytic domain (Pines, 1996). They are inactive as monomers, and activation requires not only association with positive regulatory cyclin subunit but also the phosphorylation of a conserved threonine residue (T161 in human CDK1 and T160 CDK2) by a separate protein kinase known as the CDK-activating kinase (Brown et al., 1999). In mammalian cells the activating kinase is another cyclin dependent kinase, CDK7/CyclinH associated with a third protein called MAT-1 ("ménage à trois-1"). In yeast cells this activation is performed by a single protein kinase (CAK or Civ1) (Desai et al., 1995). CDK7 phosphorylates and regulates CRK1, CDK2, CRK4 and CDK6 (Lolli and Johnson, 2005).

Cyclin activated kinases drives the cell to the next cell cyde stage where the cyclin is targeted to destruction in the cyclin destruction box by proteasome degradation after ubiquitination. In human cells two CDKs are well understood, CDK1 is activated by cyclin B in the G2/M transition (Pines and Hunter, 1994) and is involved in induction of mitosis. CDK2, is activated late in G1 and remains active until the end of G2; its major partners are cyclin E and A (Koff et al., 1992;Dulic et al., 1992). CDK2-cyclin complexes appear to be involved in the control of G1 events and DNA replication (Cardoso et al., 1993;Pagano et al., 1993;van den and Harlow, 1993). Differently from mammalian cells, yeast cells have

only one CDK (*Schizosaccharomyces pombe* cdc2 or *Saccharomyces cerevisiae* CDC28), which is responsible for transition through both G1/S and G2/M boundaries (Nurse and Bissett, 1981;Reed and Wittenberg, 1990).

In summary, the eukaryotic cell cycle can described as follows. Progression through the G1, S, G2 and M phases of the cell division cycle are regulated by CDKs. In early–mid G1, extracellular signals modulate the activation of CDK4 and CDK6 associated with D-type cyclins. These complexes phosphorylates and inactivate the retinoblastoma protein (pRb), resulting in the release of the E2F and DP1 transcription factors, which control the expression of genes required for G1/S transition and S phase progression. The CDK2–cyclin E complex is responsible for G1/S transition but also regulates centrosome duplication. During S phase, CDK2–cyclin A phosphorylates various substrates allowing DNA replication and the inactivation of G1 transcription factors. Around the S/G2 transition, CDK1 associates with cyclin A. Later, CDK1–cyclin B appears and triggers the G2/M transition by phosphorylating a large set of substrates. Phosphorylation of the 'anaphase promoting complex' by CDK1–cyclin B results in transition to anaphase and completion of mitosis. These successive waves of CDK–cyclin assembly, activation and inactivation are regulated tightly by post-translational modifications and intracellular translocations, and are coordinated and dependent on the completion of previous steps, through so-called 'checkpoint' controls (Knockaert et al., 2002).

Certain CDKs, including CDK1 and CDK2, form tight complexes with a small (9-18kDa) protein known generically as the Cdc kinase subunit (Cks). These proteins are believed to be essential for CDK function and cell cycle but their precise function is poorly understood (Pines, 1996). Cks and cyclins physically interact simultaneously and independently with CDKs. Cks proteins do not act as inhibitors or activators in the classic sense, but seem to modulate substrate choice or the extent of phosphorylation (Harper, 2001). The association of a Cks protein, in its monomeric state, with the CDK1–Cyclin B complex permits the

phosphorylation of the CDC25 phosphatase and the entry in mitosis (Patra *et al.*, 1999). CDK1–Cyclin B–CKS complex phosphorylates the sub-unit CDC27 of the anaphase promoting complex, which promotes ubiquitination of cyclin B and exit from mitosis (Patra and Dunphy, 1998) After cyclin B is ubiquitinate, Cks1 interact with the proteasome to promote proteolysis of the cyclin (Kaiser *et al.*, 1999). The human Cks1 hCKS1 associates with SKP2, a subunit of the SCF-ubiquitin-ligase complex, and favours the ubiquitination of the CKI (cdk inhibitor) $p27^{Kip1}$ and thus the G1/S transition (Pines, 1996;Nakayama et al., 2000).

Previous studies have suggested that the affinity of the interaction between CDKs and cyclins may be affected by phosphorylation at the activating threonine. According to (Desai et al., 1995), certain CDK-cyclin complexes form readily *in vitro* in the absence of other proteins or phosphorylation, whereas other complexes form only when CDK phosphorylation is allowed to occur. It is also known that CDK2-cyclin A exhibits about 0.2% of the activity of the fully activated phosphorylated binary complex (Brown *et al.*, 1999). The effect of the cyclin A bound to CDK2 is that this protein assumes a protein kinase fold with mostly β-sheet structure on the N-terminal domain that contains one helix, the C-helix, and a predominantly α- helical C terminal domain. The ATP binding site is situated at the domain-domain interface. There are no changes in cyclin A structure when bound to CDK2, but CDK2 changes to create the ATP recognition site (Brown *et al.*, 1999;Lolli *et al.*, 2005). Inactive monomeric CDK2 can not bind ATP but because the necessary residues are not properly disposed and the alignment for the triphosphate catalysis is disabled. This inactive conformation is due to the C-helix, which contains the PSTAIRE motif and the activation segment (residues 145-147 and 170-172). Thr160 in the inactive CDK2 is not correctly located (it is buried in the conserved glycine-rich loop) because of the loss of interaction between E51 (the E residue of the PSTAIRE sequence) and L33 (Desai *et al.*, 1995;Russo et al., 1996).

It is generally accepted that mammalian cells require Cdk1, Cdk2, Cdk3, Cdk4 and Cdk6, to drive cells through the cell cycle (Figure 1.2). However, since unicellular organisms such as yeasts require a single cyclin-dependent kinase, experiments that knocked out several Cdks in mice embryonic cells, showed that mammalian cells require only Cdk1 to drive the cell cycle (Santamaria et al., 2007). Cdk4–CyclinD, Cdk6–CyclinD and Cdk3–CyclinC complexes are believed to be involved in regulation of the G0–G1 transition and the early phases of G1 (in proliferating cells) by phosphorylating the pRb. Cdk2–CyclinE complexes have been also implicated in the G1–S transition. Cdk2 associates with Cyclin A during S phase. Cdk1 participates in the S–G2 and G2–M transitions by sequential binding to Cyclin A and Cyclin B. CAK, as already mentioned, phosphorylates, and presumably activates, all cell-cycle Cdks. Cdk10 and Cdk11 may be involved in mitosis, but their roles is not known. Finally, Cyclin F is probably required for entry into G1 and Cyclin G is acts in the DNA damage response during the G2–M transition (Figure 1.2) (Malumbres and Barbacid, 2005).

Cdk7 to Cdk11 are related to the cell-cycle control through activities of transcription. CAK, along with six additional subunits, forms the general transcription factor TFIIH that is involved in promoter clearance and progression of transcription. Cdk8–CyclinC and Cdk9–CyclinT complexes also regulate transcription by phosphorylating the C-terminal domain of the large subunit of RNA polymerase II. Cdk8–CyclinC complex is actually a component of the RNA polymerase holoenzyme. Cdk8–CyclinC also phosphorylates Cyclin H to inhibit CAK activity. Cdk9, by contrast, binds to Cyclin T and Cyclin K to form the P-TEFb transcription factor implicated in transcript elongation by RNA polymerase II (Malumbres *et al.*, 2005;Santamaria *et al.*, 2007).

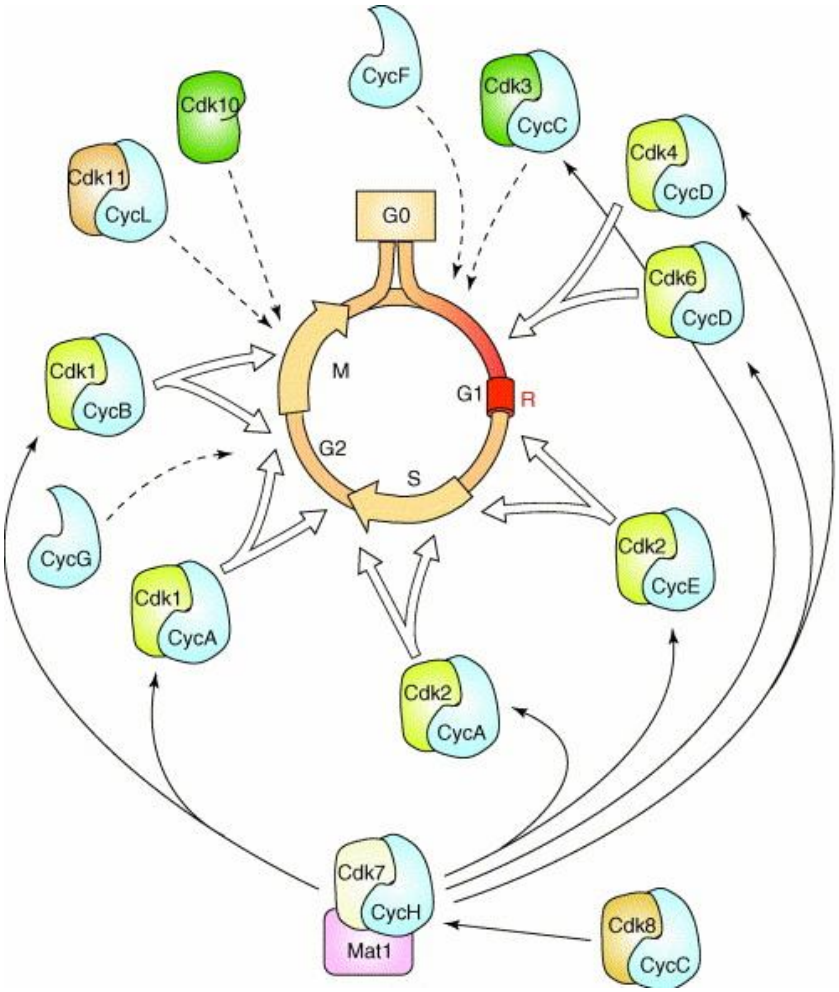


Figure 1.2 The role of CDKs in the cell cycle. Adapted from (Malumbres *et al.*, 2005). From G0 to the restriction point R, where the cells commit to mitosis, 3 different kinases are involved, Cdk3, Cdk4 and Cdk6 in complex with Cyclin C (CycC) and CycD. After the restriction point R, G1 specific CycE and Cdk2 drive the cells to S-phase where they are still active together with Cdk2-CycA complex. In G2, mitotic Cdk1-CycA (possibly CycG as well) are active. At the end of G2 and in mitosis, Cdk1 forms a complex with CycB. Cdk11-CycL and Cdk11 have also a role in mitosis. Cak or Cdk7 complex phosphorylates most of the cyclin-kinase complexes through the cycle.

Cdk5 can bind to two different activators, p35 and p39, expressed in the brain. When complexed with Cyclin D or Cyclin E, Cdk5 does not appear to have known substrates. Cdk5–p35 and Cdk5–p39 complexes phosphorylate numerous substrates involved in several aspects of transcription and neuronal functions (Malumbres *et al.*, 2005;Santamaria *et al.*, 2007).

Other protein kinases are involved in the cell cycle. Aurora kinases, like Aurora A, B and C, have their role as regulators of mammalian cell division. Aurora kinases are essential to ensure error-free cell division (Hassan *et al.*, 2001;Carmena and Earnshaw, 2003). Mammalian Polo-like kinase family is also involved in the cell cycle. Plk1, the best studied polo, specifically localizes in the centrosomes, the spindle midzone and the post-mitotic bridge, and it acts in both mitotic entry and mitotic progression (Barr *et al.*, 2004). Some family members of the NIMA family, such as Nek2, Nek6, Nek7 and Nek9, are involved in mitotic progression (Hayes *et al.*, 2006). MOB kinases, a small family of highly conserved, non-catalytic proteins that are found in all eukaryotes, play critical roles in cell-cycle regulation and function chiefly by interacting with and activating the Dbf2-related protein kinases. The functional co-dependence of the Mob and Dbf2-like proteins is similar to that of how cyclins bind and regulate Cdks (Mrkobrada *et al.*, 2006).

1.5 Proteomics and the kinome of pathogenic trypanosomatids

Six major groups of eukaryotic protein kinase have been defined on the basis of sequence similarity of the catalytic domains: AGC, CAMK, CMGC, TK, TKL, STE (Hanks and Hunter, 1995). Approximately 2% of the *L. major, T. brucei* and *T. cruzi* genomes codes for protein kinases. The analysis of these genomes (Parsons *et al.*, 2005) revealed a total of 176 protein kinases in *T. brucei*, 190 in *T. cruzi* and 199 in *L. major*, most of which are orthologous across the three species. This is approximately 30% of the number in the human host and double that of the malaria parasite, *Plasmodium falciparum*. Among the

various groups of eukaryotic protein kinases identified, the CMGC, STE and NEK groups showed an evolutionary expansion and are overrepresented in comparison to other organisms (Parsons *et al.*, 2005).

Protein kinases are classified into two superfamilies: serine/threonine kinases and protein tyrosine kinases. In trypanosomatids CMGC kinases include MAP kinases (MAPKs), dual specificity CLK and DYRK kinases and CRKs. While unicellular yeast has a single cyclin dependent kinase essential for the cell cycle (CDC28), the CRK family is relatively large in trypanosomatids with 11 members in *T. brucei* and *L. major* and 10 in *T. cruzi*. This complexity may reflect the problem of dividing a highly polarized cell with an elaborate cytoskeleton and a single mitochondrion, along with an integral link between cell cycle control and life cycle differentiation.

It was proposed that phosphorylation on tyrosine in trypanosomatids is likely to be due to the action of atypical tyrosine kinases such as Wee1 and dual-specificity kinases that can phosphorylate serine, threonine, and tyrosine (Parsons *et al.*, 2005). Multiple members of the dual specificity kinase families (DYRKs, CLKs, and STE7) are present in the trypanosomatid genomes. In yeast and higher eukaryotes Wee1 phosphorylates a conserved tyrosine residue in the ATP binding pocket of CDK1 (cdc2), inactivating the protein kinase. This mechanism is likely to be conserved in the three trypanosomatids, since there are two Wee1 family members in *L. major* and *T. cruzi* and one in *T. brucei*. In addition, CRK3, the putative functional CDK1 homologue in trypanosomatids, contains a conserved tyrosine residue in the same subdomain as the human CDK1 regulatory tyrosine (Naula *et al.*, 2005).

A large number of MAPK-related genes are also present in trypanosomatids. The parasites undergo substantial changes of temperature, pH, nutrients, and stresses during their developmental cycle. A phosphorylation signaling system that responds to those sudden

changes may be a key strategy for this group of organisms (Parsons *at al.*, 2005). MAPKs are activated by phosphorylation within the activation loop, typically both on a tyrosine and a threonine. MAP kinase kinases, which are members of the STE7 family, perform this activation. STE7 is one of the three major families of STE group kinases that are generally described as upstream regulators of MAP kinase cascades. Some of the MAPKs, like *Leishmania mexicana* LmxPK4 have being studied and found to have a role in parasite differentiation (Kuhn and Wiese, 2005). Another, LmxMKK has a role in flagellar length (Wiese et al., 2003).

Trypanosomatids lack receptor-linked tyrosine and tyrosine kinase-like kinases, although they do possess dual-specificity kinases. It was also shown that these parasites possess a large number of unique eukaryotic protein kinases that show no strong affinity to any known group. Few protein kinases with predicted transmembrane domains were identified, suggesting that receptor eukaryotic protein kinases are absent (Parsons *et al.*, 2005).

1.6 The trypanosomatid cell cycle

Trypanosomes are flagellated protists that contain a single Golgi complex (He *et al.*, 2004) as well as a large mitochondrion that hosts a single kinetoplast formed by a network of minicircle and maxicircle DNA molecules. A single flagellum connected to the kinetoplast at its basal body emerges from a flagellar pocket, an invagination of the plasma membrane (Gull, 2003). In model organisms, like yeast, the mechanisms that ensure cell division have been studied with respect to the control of cell growth, DNA replication, and mitosis, including the establishment of networks of interacting molecules expressed at different times of the cell cycle. However, much less is known about the relationship between the duplication and segregation of organelles and cell cycle progression in Kinetoplastids.

All trypanosomatids contain single-copy organelles, such as the nucleus, mitochondrion, kinetoplast (containing the mitochondrial genome), basal body and flagellum. In *T. brucei*,

the morphological events occurring during the cell cycle were described (Woodward and Gull, 1990). Morphological alterations that occur during the cell cycle of other species of trypanosomes are different as they have distinct cell shapes, kinetoplast position, and flagellum insertion (Elias et al., 2007). *Crithidia* and *Leishmania* species, for instance, do not replicate their nuclear and kinetoplast DNA in the same order as *T. brucei* (Elias *et al.*, 2007). There is no migration of the basal body and kinetoplast segregation occurs close to the nuclear mitosis. In addition, although no detailed description has been provided, the new flagellum does not attach to the old one (Briggs *et al.*, 2004).

The investigations of the molecules that regulate the cell cycle of *Leishmania* have initially identified two cdc-2 related protein kinases, CRK1 and a CRK3 (Mottram et al., 1993;Mottram and Grant, 1996a). The *L. mexicana* CRK3 gene that encodes for a cdc2-related protein kinase with activity towards histone H1 at G2:M (Grant et al., 1998), is encoded by an essential gene and is post translationally regulated in a stage-specific manner, being active in dividing cells (promastigotes and amastigotes) but not in non-dividing cells (metacyclic promastigotes) (Grant et al., 2004).

The data already published about *Leishmania* CRK3 (Grant *et al.*, 1998;Hassan *et al.*, 2001) shows that CRK3 encodes a 356kDa cdc-2 related kinase with activity towards histone H1 that is essential for the progression of *L. mexicana* cell cycle. It also has 54% sequence identity with human cyclin dependent kinase cdk1 and 78% identity with *T. brucei* CRK3. The trypanosomatid CRK3 have an unusual, poorly conserved 19-amino acid N-terminal extension not present in human cdc2. CRK3 is a single copy, and there is a 5-fold higher mRNA presence in the replicative promastigote life cycle stage than in non-dividing metacyclic form or mammalian amastigote form.

Despite the existence of a large number of CDK family members (named CRK for cdc2-related kinase), only 2 have been shown to be essential for cell cycle progression. CRK3 in

complex with the CYC6 mitotic cyclin is essential for G2/M phase progression and is the functional homologue of CDK1 (Hassan *et al.*, 2001;Hammarton *et al.*, 2003a;Tu *et al.*, 2004;Tu and Wang, 2005). CRK3 in complex with CYC2 is essential for G1 progression (Li *et al.*, 2003b;Hammarton et al., 2004;Gourguechon *et al.*, 2007). A CYC2 cyclin and a CYC6 cyclin control the cell cycle of the procyclic form of *T. brucei* (Li et al., 2003a), while *TbCRK1* is also an essential gene required for G1 phase progression (Tu *et al.*, 2004;Tu *et al.*, 2005). However, the roles of CRKs in the cell cycle are complex, with functional differences between bloodstream and procyclic form *T. brucei* as revealed by RNAi knockdown studies.

CRK7 has the highest level of sequence identity to CDK7 of mammals. CDK7, in complex with cyclin H and MAT1, is a CDK-activating kinase (CAK) that phosphorylates the T-residue of CDKs (e.g., T160 of human CDK1). No cyclin H or MAT1 orthologues can be identified in trypanosomatids based on sequence, so it remains to be determined if CRK7 is a functional cyclin-dependent kinase or indeed if it has CAK activity. However, many CRKs, including CRK1, 2, 3, 6, 7, 8, 9 and 12, have a conserved T-loop residue, suggesting that the CRKs might be activated *in vivo* by a CAK activity (Naula *et al.*, 2005)

Other protein kinases like polo kinases have been studied in trypanosomes. *T. brucei* polo-like-kinase (TbPLK) can complement the temperature-sensitive *S. cerevisiae cdc5-1* mutant. RNAi of *PLK* in procyclic *T. brucei* inhibited growth, indicating a role for PLK in the initiation of cytokinesis in this life cycle stage (Kumar and Wang, 2006). In another study, downregulation of PLK results in an earlier cell cycle defect in basal body duplication and delay in kDNA replication preventing thus cytokinesis (Hammarton *et al.*, 2007).

1.7 Kinase inhibitors

Cyclin-dependent protein kinases are attractive targets for drug discovery and efforts have led to the identification of novel Cdk selective inhibitors in the development of treatments for cancers, neurological disorders, and infectious diseases. CDKs from parasites have been identified and several are considered potential drug targets. A series of chemical inhibitors, which display various degrees of CDK selectivity, have been identified, including olomoucine (Havlicek *et al.*, 1997), roscovitine (Meijer *et al.*, 1997), purvalanol (Gray *et al.*, 1998), flavopiridol (Losiewicz et al., 1994), butyrolactone (Kitagawa *et al.*, 1993), indirubins (Hoessel et al., 1999), and paullones (Zaharevitz *et al.*, 1999). All these inhibitors act by competing with ATP for binding at the catalytic site. About 70 different kinases have had their structures determined and these databases are being used for developing more specific inhibitors with some limitations (Thaimattam *et al.*, 2007). Kinase inhibitors such as dasatinib are currently used for the treatment of cancer and several of these kinase inhibitors are specific for tyrosine kinase (Thaimattam *et al.*, 2007).

As cytotoxic drugs, Cdk inhibitors have more effect on tumor cells, which are rapidly dividing, than on normal quiescent cells. Considering kinetoplastid parasites as the undesired proliferating cell instead of a tumor, the same effect could be obtained against parasitic diseases using Cdk inhibitors (Grant *et al.*, 2004). It has been shown to be possible to obtain Cdk specific inhibitors. This has been achieved through the development of inhibitors that are capable of differentially inhibit Cdk families. More studies are needed to discover, design, or develop inhibitors that are selective for parasite protein kinases of the CDK family. The *Leishmania* kinase CRK3 was studied as a novel antileishmanial drug target (Grant *et al.*, 2004). In a kinase inhibitor screening, only 3 compounds had similar IC_{50} to control human Cdk1-Cyclin B. All other tested compounds showed a range of variation suggesting that it is possible to specific inhibit cell proliferation with Cdk inhibitors.

1.8 Aims of the project

Based on the above the aim of this project was to study cell cycle related proteins, cyclins and kinases from trypanosomatid parasites *Leishmania* and *T. brucei in vitro* and *in vivo.* Specifically:

To generate an active CRK3 kinase from *T. brucei* and *Leishmania in vitro*

To characterize biochemically the CRK3:CYCA complex

To define the role of the phosphorylation of the Thr178 on the kinase activity of CRK3

To study the role of Cks1 protein in the activity of CRK3:CYCA complex

Try to generate a system to identify cyclin:kinase complexes

To try to identify other CRK/cyclin pairs in *L. major*

To tag CYCA and study its function *in vivo*

To tag CRK6 and study its function *in vivo*

Chapter 2

General Methods

2.1 Genomic DNA preparation from *Leishmania*

10 ml of log phase *Leishmania* culture was pelletted for 10 min at 1000g. Pellets were resuspended in lysis buffer from a gDNA extraction kit (Qiagen) and extraction procedures were performed according to manufacture's instructions. The gDNA extracted from *L. mexicana* and *L. major* was used for amplifying the genes of the proteins of interest. The same extraction procedure was also used for obtaining *T. brucei* gDNA.

2.2 Cloning

For the cloning of the following proteins, the same general procedure was used, which is described below. PCR reactions were set up in a final volume of 50µl using 10mM oligos, 0.5 Unit Thermozyme (Invitrogen), 1µl genomic DNA, 10X PCR mix and water. Thermocycling conditions consisted of 1min at 94°C followed by 25 cycles at 94°C for 30 sec, 65°C for 1 min and 72°C for 90 sec and was completed with 10 min at 72°C. PCR products (1µl) were checked by electrophoresis on 1% agarose gel stained with ethidium bromide. PCR product purification was then carried out using the Qiaquick Gel Extraction kit (Qiagen). The purified PCR products were again visualized on 1% agarose gel stained with ethidium bromide to confirm the purification quality and ligated into PGEM-T plasmid (Novagen). Ligation into pGEM-T and subsequent vectors was performed as follows: plasmid and DNA insert were mixed in a ratio of 3:1 respectively in a 10µl reaction containing 1X T4 ligase buffer and 1Unit of T4 ligase (New England Biolabs) overnight at room temperature. Ligation reactions were then used to transform DH5α *E. coli* cells by heat shock (30 min on ice/ 30 sec at 42^{o}C). Transformation reaction was then plated on L. agar plates containing the appropriate antibiotic necessary for selection of the clones. Positive clones were selected and analysed by restriction enzyme digestions.

2.2.1 L. major CYC3 (LmCYC3his)

PCR amplification of CYC3 (LmjF30.0080) was performed using genomic DNA (gDNA) from *L. major*, oligos OL1765 and OL1762 (Table 2.1) and Invitrogen Thermozyme polymerase. The PCR product was gel purified and ligated in the pGEM-T vector generating pGL1266. The sequence of the insert was confirmed by alignment with LmjF30.0080. The PCR product was excised from pGL1266 using *Bam*HI and *Nde*I restriction sites generated by the PCR oligos and subcloned in the expression vector pET-15b+, which was pre-digested with *Bam*HI and *Nde*I generating pGL1350. This plasmid was used to express LmCYC3his and an affinity purification of this protein was also attempted.

2.2.2 L. major CYC7 (LmCYC7his)

PCR amplification of LmjF30.3630 was performed using gDNA from *L. major*, oligos OL 1767 and OL 1768 (Table 2.1) and Invitrogen Thermozyme polymerase. The PCR product was gel purified and ligated in the pGEM-T vector generating pGL1267. The sequence of the insert was confirmed by alignment with LmjF30.0080. The PCR product was excised from pGL1267 using *Bam*HI and *Nde*I restriction sites generated by the PCR oligos and subcloned in the expression vector pET-15b+, which was pre-digested with *Bam*HI and *Nde*I generating pGL1335. This plasmid was used to express and affinity purify LmCYC7his.

2.2.3 L. major CYC9 (LmCYC9his)

PCR amplification of LmjF32.0760 was performed using gDNA from *L. major*, oligos OL1770 and 1771 (Table 2.1) and Invitrogen Thermozyme polymerase. The PCR product was gel purified and ligated in the pGEM-T vector generating pGL1253. The sequence of the insert was confirmed by alignment with LmjF32.0760. The PCR product was excised

from pGL1253 using *Bam*HI and *Nde*I restriction sites generated by the PCR oligos and subcloned in the expression vector pET-15b+, which was pre-digested with *Bam*HI and *Nde*I generating pGL1336. This plasmid was used to express and affinity purify LmCYC9his.

2.2.4 L. major CYC10 (LmCYC10his)

PCR amplification of LmjF24.1890 was performed using gDNA from *L. major*, oligos OL1772 and OL1773 (Table 2.1) and Invitrogen Thermozyme polymerase. The PCR product was gel purified and ligated in the pGEM-T vector generating pGL1268. The sequence of the insert was confirmed by alignment with LmjF24.1890. The PCR product was excised from pGL1268 using *Bam*HI and *Nde*I restriction sites generated by the PCR oligos and subcloned in the expression vector pET-15b+, which was pre-digested with *Bam*HI and *Nde*I generating pGL1337. This plasmid was used to express and affinity purify this protein LmCYC10his.

2.2.5 L. major CRK1 (LmCRK1his)

PCR amplification of LmjF21.1080 was performed using gDNA from *L. major*, oligos OL1783 and OL1784 (Table 2.1) and Invitrogen Thermozyme polymerase. The PCR product was gel purified and ligated in the pGEM-T vector generating pGL1254. The sequence of the insert was confirmed by alignment with LmjF21.1080. The PCR product was excised from pGL1254 using *Bam*HI and *Nde*I restriction sites generated by the PCR oligos and subcloned in the expression vector pET-15b+, which was pre-digested with *Bam*HI and *Nde*I generating pGL1338. This plasmid was used to express and affinity purify LmCRK1his.

2.2.6 L. major CRK2 (LmCRK2his)

PCR amplification of LmjF05.0550 was performed using gDNA from *L. major*, oligos OL1785 and OL1786 (Table 2.1) and Invitrogen Thermozyme polymerase. The PCR product was gel purified and ligated in the pGEM-T vector generating pGL1269. The sequence of the insert was confirmed by alignment with LmjF05.0550. The PCR product was excised from pGL1269 using *Bam*HI and *Nde*I restriction sites generated by the PCR oligos and subcloned in the expression vector pET-15b+, which was pre-digested with *Bam*HI and *Nde*I generating pGL1339. This plasmid was used to express and affinity purify LmCRK2his.

2.2.7 L. major CRK3 (LmCRK3his)

PCR amplification of LmjF36.0550 was performed using gDNA from *L. major*, oligos OL1787 and OL1789 (Table 2.1) and Invitrogen Thermozyme polymerase. The PCR product was gel purified and ligated in the pGEM-T vector generating pGL1255. The sequence of the insert was confirmed by alignment with LmjF36.0550. The PCR product was excised from pGL1255 using *Bam*HI and *Nde*I restriction sites generated by the PCR oligos and subcloned in the expression vector pET-15b+, which was pre-digested with *Bam*HI and *Nde*I generating pGL1340. This plasmid was used to express and affinity purify LmCRK3his.

2.2.8 L. major CRK4 (LmCRK4his)

PCR amplification of LmjF16.0990 was performed using gDNA from *L. major*, oligos OL1789 and OL1790 (Table 2.1) and Invitrogen Thermozyme polymerase. The PCR product was gel purified and ligated in the pGEM-T vector generating pGL1270. The sequence of the insert was confirmed by alignment with LmjF16.0990. The PCR product was excised from pGL1270 using *Bam*HI and *Nde*I restriction sites generated by the PCR

oligos and subcloned in the expression vector pET15b+, which was pre-digested with *Bam*HI and *Nde*I generating pGL1616. This plasmid was used to express and affinity purify LmCRK4his.

2.2.9 L. major CRK6 (LmCRK6his)

PCR amplification of LmjF27.0560 was performed using gDNA from *L. major*, oligos OL1791 and OL1792 (Table 2.1) and Invitrogen Thermozyme polymerase. The PCR product was gel purified and ligated in the pGEM-T vector generating pGL1271. The sequence of the insert was confirmed by alignment with LmjF27.0560. The PCR product was excised from pGL1271 using *Bam*HI and *Nde*I restriction sites generated by the PCR oligos and subcloned in the expression vector pET-15b+, which was pre-digested with *Bam*HI and *Nde*I generating pGL1341. This plasmid was used to express and affinity purify LmCRK6his.

2.2.10 L. major CRK7 (LmCRK7his)

PCR amplification of LmjF26.0040 was performed using gDNA from *L. major*, oligos OL1793 and OL1794 (Table 2.1) and Invitrogen Thermozyme polymerase. The PCR product was gel purified and ligated in the pGEM-T vector generating pGL1256. The sequence of the insert was confirmed by alignment with LmjF26.0040. The PCR product was excised from pGL1256 using *Bam*HI and *Nde*I restriction sites generated by the PCR oligos and subcloned in the expression vector pET-15b+, which was pre-digested with *Bam*HI and *Nde*I generating pGL1349. This plasmid was used to express and affinity purify LmCRK7his.

2.2.11 L. major CRK8 (LmCRK8his)

PCR amplification of LmjF11.0110 was performed using gDNA from *L. major*, oligos OL1795 and OL1796 (Table 2.1) and Invitrogen Thermozyme polymerase. The PCR product was gel purified and ligated in the pGEM-T vector generating pGL1272. The sequence of the insert was confirmed by alignment with LmjF11.0110. The PCR product was excised from pGL1272 using *Bam*HI and *Nde*I restriction sites generated by the PCR oligos and subcloned in the expression vector pET-15b+, which was pre-digested with *Bam*HI and *Nde*I generating pGL1342. This plasmid was used to express and affinity purify LmCRK8his.

2.2.12 Non-tagged L. mexicana CRK3 (LmCRK3)

L. mexicana CRK3 from pGL751 was excised using *Nde*I/*Bam*H1 restrictions sites and cloned into *Nde*I/*Bam*HI of pET21a+ generating pGL1072. This plasmid expresses a non-his-tagged version of CRK3 to be used in binding assays.

2.2.13 T. brucei CYC6 box (TbCYC6boxGST)

PCR amplification was performed using *T. brucei* Eatro 795 gDNA, oligos OL1413 and OL1414 (Table 2.1) and Invitrogen Thermozyme polymerase. The PCR product was gel purified and cloned in the pGEM-T vector generating pGL979. The sequence of the insert was confirmed by alignment with Tb11.01.8460. The PCR product was excised from pGL979 using *Bam*HI and *Sal*I restriction sites generated by the PCR oligos and subcloned in the expression vector pGEX5X-1, which was pre-digested with *Bam*HI and *Sal*I generating pGL980. This plasmid was used to express and affinity purify TbCYC6box protein.

2.2.14 T. brucei CRK3 (TbCRK3his)

PCR amplification was performed using *T. brucei* Eatro 795 gDNA, oligos OL1412 and OL427 (Table 2.1) and Invitrogen Thermozyme polymerase. The PCR product was gel purified and ligated in the pGEM-T vector generating pGL977. The sequence of the insert was confirmed by alignment with Tb10.70.2210. The PCR product was excised from pGL977 using *Bam*HI and *Nde*I restriction sites generated by the PCR oligos and subcloned in the expression vector pET-15b+, which was pre-digested with *Bam*HI and *Nde*I generating pGL1036. This plasmid was used to express and affinity purify TbCRK3his.

2.2.15 T. brucei CKS1 (Tbp12^{CKS1}his)

The *T. brucei* CKS1 (Tb11.01.8085), an accessory protein involved in the cyclin:kinase complex, was cloned into the pET15b with an N-terminal histidine tag. PCR amplification was carried out using *T. brucei* gDNA, oligos OL1260 and OL861 (Table 2.1) and Invitrogen Thermozyme. The PCR product was gel purified and cloned into the pGEM-T vector creating pGL975. The sequence for Tbp12^{CKS1}his was confirmed by alignment with Tb11.01.8085. The PCR product contained *Xho*I and *Nde*I restriction sites that were used to remove the fragment from pGL975 and subcloned in the expression vector pET15b to give pGL976. This plasmid was used to express and purify Tbp12^{CKS1}his.

Table 2.1 Primer sequences

Description	Primer	Primer sequence 5' to 3'
TbCYC6 cyclin box sense primer for cloning into pGEX5X-1	**OL1413**	**GAAAGGATCCCCGTAGGTACGGCAGGGAA**
TbCYC6 cyclin box antisense primer for cloning into pGEX5X-1	**OL1414**	**GTTTGTCGACTCACATTTGTGTCTGTTGAGA**
5' flank of TbCYC6 locus sense primer (NotI)	**OL1467**	**GCGGCCGCACGCCAGATGTCGAAATTCC**
5' flank of TbCYC6 locus antisense primer (XbaI)	**OL1468**	**TCTAGACGTTCGTTCGTAACTCCACT**
3' flank of TbCYC6 locus sense primer (ApaI)	**OL1469**	**GGGCCCTGATTGGTTTACTTCGTTAC**
3' flank of TbCYC6 locus antisense primer (ApaI/EcoRV)	**OL1470**	**GGGCCCGATATCCTTTGGAACTCCTCTTTCTC**
*L.major*CYC6 sense primer for cloning into pET15b (Nde-I)	**OL1515**	**GCATATGTTCGTGGAACGAGAGCGGCAGG**
*L.major*CYC6 antisense primer for cloning into pET15b (Xho-I)	**OL1516**	**GCTCGAGTCACAGCGCCGGCAGCTCGTTAGGC**
Sense primer for *L.major*CYCA 5'flank (HindIII)	**OL1750**	**GAAGCTTGGTTGGTGCGATTCATCCC**
Antisense primer for *L.major*CYCA 5'flank (SalI)	**OL1751**	**GGTCGACTTTGACCGCCGTGCTTCTA**
Sense primer for *L.major*CYCA 3'flank (SmaI)	**OL1752**	**GCCCGGGTTAGTGTCTGCTTTCGTGG**
Antisense primer for *L.major*CYCA 3'flank (BglII)	**OL1753**	**AGATCTACAGCATGGGCACAACGCC**
Sense primer for *L.major*CYCA (NdeII) C-term TAP TAG	**OL1754**	**GCATATGGCGGTCCCACTGCGAATG**
Antisense primer for *L.major*CYCA (KpNI) N-term TAP TAG	**OL1755**	**CGGTACCCGCAGAAGTTGAAATGAAAG**
Sense primer for *L.major*CYC6 (NdeII) C-term TAP TAG	**OL1756**	**TCATATGTTCGTGGAACGAGAGCGG**
Antisense primer for *L.major*CYC6 (KpNI) N-term TAP TAG	**OL1757**	**TGGTACCCAGCGCCGGCAGCTCGTTAG**
Sense primer for *L.major*CYC3 (NdeI)	**OL1765**	**GCATATGTCGACCGATCTCGCAATTGTGACGACGG**
Antisense primer for *L.major*CYC3 (XhoI)	**OL1766**	**GCTCGAGGGACAGTCTGCGATGAAAGCAGTAGCAC**

Sense primer for *L.major*CYC7 (NdeI)	OL1767	CCATATGCCTTTGCGAGCTTGCGTATCCAGG
Antisense primer for *L.major*CYC7 (XhoI)	OL1768	GCTCGAGTCAGACCTCTCCGGAGGGAAGTGAAAAAG
Sense primer for *L.major*CYC9 (NdeI)	OL1770	CCATATGTCGTCAAGCCCATTGTCGCCGGTG
Antisense primer for *L.major*CYC9 (XhoI)	OL1771	GCTCGAGTCACTGCTCTGACATCTTCATGAGTGG
Sense primer for *L.major*CYC10 (NdeI)	OL1772	GCATATGCAGATTGGCTTAGCGGGGTGCAGCAG
Antisense primer for *L.major*CYC10 (XhoI)	OL1773	GCTCGAGCTACACACGGGGCTGATCTGGAGGTGGAGG
Sense primer for *L.major*CRK1 (NdeI)	OL1783	CCATATGACCAGCCGGTACGAGCGGCAGGAGAAGATC
Antisense primer for *L.major*CRK1 (BamHI)	OL1784	CGGATCCCTAAAACTGGAGGCTAAAGTACGGGTG
Sense primer for *L.major*CRK2 (NdeI)	OL1785	CCATATGCGGAGCAGCGGCCCCACCCCAGCGC
Antisense primer for *L.major*CRK2 (BamHI)	OL1786	CGGATCCTTACGACTGCTGCTGCTGCTGCTGCTG
Sense primer for *L.major*CRK3 (NdeI)	OL1787	CCATATGTCTTCGTTTGGCCGTGTTACCGCCC
Antisense primer for *L.major*CRK3 (BamHI)	OL1788	CGGATCCCTACCAGCGAAGGTCACTGAACCACGGG
Sense primer for *L.major*CRK4 (NdeI)	OL1789	CCATATGTCGACGGCGGGTCGGTACAAGCACG
Antisense primer for *L.major*CRK4 (BamHI)	OL1790	CGGATCCTCATAGCAAGTGGCAGGCCTCCATCGTC
Sense primer for *L.major*CRK6 (NdeI)	OL1791	CCATATGTCCGCGTCAGTGAACGACTTGGATG
Antisense primer for *L.major*CRK6 (BamHI)	OL1792	CGGATCCCTACGCATCCTTCATAAAGGGGTGTTCC
Sense primer for *L.major*CRK7 (NdeI)	OL1793	CCATATGGACAAGTACGCGTTGGGGCCGGTTATC
Antisense primer for *L.major*CRK7 (BamHI)	OL1794	CGGATCCTCATGCACGCAGCAAGGTATCTGAGAG

Sense primer for *L.major*CRK8 NdeI)	**OL1795**	CCATATGGGAGGGGAACTGGATAACCAGAAC
Antisense primer for *L.major*CRK8 (BamHI)	**OL1796**	CGGATCCTCAATGCTCCAGCTCCTTCCGCTTGACC
Sense primer for *L.major*CYCA HA TAG amplification from pGL630	**OL1896**	GGGGATCCTCACTCGAGCGCAGAAGTTGAA
Antisense for *L.major*CYCA HA TAG amplification from pGL630	**OL1897**	GGCCCGGGATGTACCCCTACGACGTCCCGGACTAT GCCATGGCGGTCCCACTGCGAATG
Sense primer for *L.mexicana*CYCA HA TAG (SmaI) C-terminal	**OL1935**	CCCCGGGATGGCGGTCCCACTGCGAATGAGGA
Antisense primer for *L.mexicana*CYCA HA TAG (BamI) C-terminal	**OL1936**	TGGATCCTCAGGCATAGTCCGGGACGTCGTAGGGG TACGCAGAAGTTGAAATGAAAGGAAATTC
Sense primer for *L.mexicana*CYCA HA TAG (SmaI) N-terminal	**OL1937**	CCCCGGGATGTACCCCTACGACGTCCCGGACTATG CCATGGCGGTCCCACTGCGAATG
Antisense primer for *L.mexicana*CYCA HA TAG (BamI) N-terminal	**OL1938**	GTGGATCCTCACGCAGAAGTTGAAATGAAAGG
Sense primer for *L.major*CRK6 HA TAG (SmaI) C-terminal	**OL1939**	CCCCGGGATGTCCGCGTCAGTGAACGAC
Antisense primer for *L.major*CRK6 HA TAG (BamI) C-terminal	**OL1940**	TGGATCCTCAGGCATAGTCCGGGACGTCGTAGGGG TACGCATCCTTCATAAAGGGGTTG
Sense primer for *L.major*CRK6 HA TAG (SmaI) N-terminal	**OL1941**	CCCCGGGATGTACCCCTACGACGTCCCGGACTATG CCATGTCCGCGTCAGTGAACGAC
Antisense primer for *L.major*CRK6 HA TAG (BamI) N-terminal	**OL1942**	TGGATCCCTACGCATCCTTCATAAAGGG
Sense primer for *L.mexicana*CYCA HA TAG (XhoI) N-terminal	**OL1943**	CCTCGAGATGGCGGTCCCACTGCGAATG
Antisense primer for *L.mexicana*CYCA HA TAG (NotI) N-terminal	**OL1944**	CGCGGCCGCTCAGGCATAGTCCGGGACGTCGTAG GGGTACGCAGAAGTTGAAATGAAAG
Sense primer for *L.mexicana*CYCA HA TAG (XhoI) C-terminal	**OL1945**	CCTCGAGATGTACCCCTACGACGTCCCGGACTATG CCATGGCGGTCCCACTGCGAATG
Antisense primer for *L.mexicana*CYCA HA TAG (NotI) C-terminal	**OL1946**	CGCGGCCGCTCACGCAGAAGTTGAAATGAAAGG
Sense primer for *L.major*CRK6 HA TAG (XhoI) N-terminal	**OL1947**	CCTCGAGATGTACCCCTACGACGTCCCGGACTATG CCATGTCCGCGTCAGTGAACG
Antisense primer for *L.major*CRK6 HA TAG (NotI) N-terminal	**OL1948**	CGCGGCCGCCTACGCATCCTTCATAAAGGGG
Sense primer for *L.major*CRK6 HA TAG (XhoI) C-terminal	**OL1949**	CCTCGAGATGTCCGCGTCAGTGAACG

Antisense primer for *L.major*CRK6 HA TAG (NotI) C-terminal	**OL1950**	CGCGGCCGCTCAGGCATAGTCCGGGACGTCGTAG GGGTACGCATCCTTCATAAAGGGG
Sense primer for *L.major*CYCA knockout 3'flank detection (pGL1249)	**OL2058**	GACTGTCGGGCGTAC
Antisense primer for *L.major*CYCA knockout 3'flank detection (CYCA knockout locus)	**OL2059**	CAACGCGTCTATTTC
Sense primer for *L.major*CYCA knockout 5'flank detection (pGL1249)	**OL2060**	CTGCGTGTCAATGCC
Antisense primer for *L.major*CYCA knockout 5'flank detection (CYCA knockout locus)	**OL2061**	GTCGCGGTGAGTTCAG
Sense primer for pGL1250 mutagenesis	**OL2132**	CACTTGATGATGACGAACATATGGCGGTC
Antisense primer for pGL1250 mutagenesis	**OL2133**	GACCGCCATATGTTCGTCATCATCAAGTG
Sense primer for pGL955 mutagenesis	**OL2134**	CACTTGATGATGACGAACATATGGCGGTCC
Antisense primer for pGL955 mutagenesis	**OL2135**	GGACCGCCATATGTTCGTCATCATCAAGTG
Sense primer for *L.mexicana*CYCA Adds NcoI site	**OL2152**	GATCCATGGATGGCGGTCCCACTGC
Antisense primer for *L.mexicana*CYCA Adds XhoI site	**OL2153**	GCTCGAGCGCAGAAGTTGAAATG
Antisense primer for *L.major*CYC6 HA TAG Adds XhoI site	**OL2154**	CCGCGGCCGCTCAGGCATAGTCCGGGACGTCGTAG G
Sense primer for *L.major*CYC6 HA TAG Adds NotI site	**OL2155**	GGTACAGCGCCGGCAGCTCGTTAG CCTCGAGATGTTCGTGGAACGAGAG
Sense primer for *L.major*CYC6 HA TAG Adds XhoI site	**OL2156**	CCTCGAGATGTACCCCTACGACGTCCCGGACTATG C
Antisense primer for *L.major*CYC6 HA TAG Adds NotI site	**OL2157**	CATGTTCGTGGAACGAGAGC CGCGGCCGCTCACAGCGCCGGCAG

2.3 Protein purification

2.3.1 L. mexicana (LmeCRK3his)

Plasmid pET28a+ (pGL751) containing LmeCRK3his (Grant *et al.*, 1998) was used to express this protein in *E. coli* BL21 DE3 pLYS cells. This expression vector added a poly-his tag to the C-terminal end of the protein and allows purification using Qiagen Ni-NTA agarose beads. For LmeCRK3his purification, BL21 DE3 *E. coli* cells were transformed with plasmid pGL751 and grown to 0.6 O.D. at 37^{o}C. Protein expression was induced at 19^{o}C over night using 150µM IPTG. Cells were centrifuged and resuspended in 40 ml of ice cold PBS pH 7.4 incubated with DNAse-I (0.1mg/ml) and Lysozyme (0.1mg/ml) for 60 min on ice and sonicated 5 x 15 sec (1 sec on/1 sec. off). The lysate was centrifuged 15000g for 20 min. The supernatant was called "Soluble aliquot" and pellet "Insoluble aliquot". To the Soluble aliquot 200 µl of Ni-NTA-agarose beads was added and incubated mixing gently for 20 min at 4^{o}C temperature and centrifuged at 1000g for 5 min at 4^{o}C. The column was washed 2 x 40 ml ice cold PBS pH 7.4 imidazole 20mM and eluted in 100 µl fractions with 100mM NaPi pH 7.4, 10mM NaCl, 0.5M imidazole. 10 µl of elution fraction was mixed to 10µL 2X Laemmli protein buffer and the total volume of 20 µl was loaded on a 12% SDS-PAGE gel. The gel was transferred to membrane and Western was performed using mouse his tag monoclonal antibody to detect and to confirm purification of the correct size protein.

2.3.2 L. mexicana CYCA (LmeCYCAhis)

For LmeCYCAhis purification, BL21 DE3 pLYS *E. coli* cells were transformed with plasmid pGL630. Cells were grown to 0.6 O.D. at 37^{o}C and induced for protein expression at 19^{o}C over night using 5mM IPTG. The same procedure used for LmeCRK3his was also used for LmeCYCAhis.

2.3.3 S. pombe (Civ1-GST)

Yeast Civ1-GST was obtained from a pGEX construct (pGL716) (Brown *et al.*, 1999). For protein expression of Civ1 BL21 DE3 pLys *E. coli* strain was used for transformation with plasmid pGL716. Cell culture was grown to 0.6 O.D. at 37°C and induced for protein expression at 21°C over night using 0.4mM IPTG. Cells were centrifuged and resuspended in ice cold PBS pH 7.4 incubated with DNAse-I (0.1mg/ml) and Lysozyme (0.1mg/ml) for 60 min. on ice and sonicated 5 x 15sec (1sec. on/1sec. off). Lysate was centrifuged 15000g for 20 min, supernatant was called Soluble aliquot and pellet Insoluble aliquot. To the Soluble aliquot was added 200 µl of glutatione-Sepharose column and incubated mixing gently for 30 min at 4°C to allow binding. Lysates were then centrifuged at 1000g for 5 min and resin column was washed 2 times with PBS 7.4 and eluted in 100 µl fractions of elution solution, 50mM Tris-Cl pH 8.0, 20mM glutathione. 10 µl of elution fraction was mixed with10 µl Laemmli protein buffer 2x and the total volume of 20 µl was loaded on a 12% SDS-PAGE gel.

2.3.4 L. major CYC3 (LmCYC3his)

For LmCYC3his purification, BL21 DE3 pLYS *E. coli* cells were transformed with plasmid pGL1350. Cells were grown to 0.6 O.D. at 37°C and induced for protein expression at 19°C over night using 5mM IPTG. The same procedure used for LmeCRK3his was also used for LmCYC3his.

2.3.5 L. major CYC7 (LmCYC7his)

For LmCYC7his purification, BL21 DE3 pLYS *E. coli* cells were transformed with plasmid pGL1335. Cells were grown to 0.6 O.D. at 37°C and induced for protein expression at 19°C over night using 5mM IPTG. The same procedure used for LmeCRK3his was also used for LmCYC7his.

2.3.6 L. major CYC9 (LmCYC9his)

For LmCYC9his purification, BL21 DE3 pLYS *E. coli* cells were transformed with plasmid pGL1336. Cells were grown to 0.6 O.D. at 37°C and induced for protein expression at 19°C over night using 1mM IPTG. The same procedure used for LmeCRK3his was also used for LmCYC9his.

2.3.7 L. major CYC10 (LmCYC10his)

For LmCYC10his purification, BL21 DE3 pLYS *E. coli* cells were transformed with plasmid pGL1337. Cells were grown to 0.6 O.D. at 37°C and induced for protein expression at 19°C over night using 5mM IPTG. The same procedure used for LmeCRK3his was also used for LmCYC10his.

2.3.8 L. major CRK1 (LmCRK1his)

For LmCRK1his purification, BL21 DE3 pLYS *E. coli* cells were transformed with plasmid pGL1338. Cells were grown to 0.6 O.D. at 37°C and induced for protein expression at 19°C over night using 1mM IPTG. The same procedure used for LmeCRK3his was also used for LmCRK1his.

2.3.9 L. major CRK2 (LmCRK2his)

For LmCRK2his purification, BL21 DE3 pLYS *E. coli* cells were transformed with plasmid pGL1339. Cells were grown to 0.6 O.D. at 37°C and induced for protein expression at 19°C over night using 1mM IPTG. The same procedure used for LmeCRK3his was also used for LmCRK2his.

2.3.10 L. major CRK3 (LmCRK3his)

For LmCRK3his purification, BL21 DE3 pLYS *E. coli* cells were transformed with plasmid pGL1340. Cells were grown to 0.6 O.D. at 37°C and induced for protein expression at 19°C over night using 1mM IPTG. The same procedure used for LmeCRK3his was also used for LmCRK3his.

2.3.11 L. major CRK4 (LmCRK4his)

For LmCRK4his purification, BL21 DE3 pLYS *E. coli* cells were transformed with plasmid pGL1616. Cells were grown to 0.6 O.D. at 37°C and induced for protein expression at 19°C over night using 1mM IPTG. The same procedure used for LmeCRK3his was also used for LmCRK4his.

2.3.12 L. major CRK6 (LmCRK6his)

For LmCRK6his purification, BL21 DE3 pLYS *E. coli* cells were transformed with plasmid pGL1341. Cells were grown to 0.6 O.D. at 37°C and induced for protein expression at 19°C over night using 1mM IPTG. The same procedure used for LmeCRK3his was also used for LmCRK6his.

2.3.13 L. major CRK7 (LmCRK7his)

For LmCRK7his purification, BL21 DE3 pLYS *E. coli* cells were transformed with plasmid pGL1349. Cells were grown to 0.6 O.D. at 37°C and induced for protein expression at 19°C over night using 1mM IPTG. The same procedure used for LmeCRK3his was also used for LmCRK7his.

2.3.14 L. major CRK8 (LmCRK8his)

For LmCRK8his purification, BL21 DE3 pLYS *E. coli* cells were transformed with plasmid pGL1342. Cells were grown to 0.6 O.D. at 37°C and induced for protein expression at 19°C over night using 1mM IPTG. The same procedure used for LmeCRK3his was also used for LmCRK8his.

2.3.15 L. mexicana CRK3/CYC6his complex

The expression of non-tagged CRK3 from pGL1072 was done when BL21 DE3 pLys cells were grown to 0.6 O.D. at 37°C and induced for protein expression at 19°C over night using IPTG at 2.5mM. The expression of L. major CYC6 was made from pGL1218 (Rod Walker PhD thesis) to transform *E. coli* BL21 DE3 pLYS and grow to 0.6 O.D at 37°C and induced for protein expression at 19°C overnight using IPTG at 5mM. Around 500 ml of *E. coli* culture of each different culture was centrifuged and mixed just prior to sonication in Sonication Buffer (SB, 50mM NaH_2PO_4 + 300mM NaCl pH 8.0). The sonication product was centrifuged at 20.000g for 15 minutes, filtered at 0.22µm and loaded in pre-equilibrated POROS MC Column (Ni^{2+} charged) for His-tagged protein purifications. Column was washed in SB + 50mM Imidazole and eluted in SB + 500mM Imidazole. The elutions containing the proteins were pooled together and re-purified by Ion-Exchange Chromatography. The pooled fractions are first passed through a desalting column (PD10, Biorad), eluted in 3.5ml of Buffer A (Tris/Cl + 5mM EDTA pH 8.0). This elution is then loaded into Ion-Exchange column (BIORAD, UNO-Q1). The purified complex is recovered in the flow through and analysed on Coomassie gel.

2.3.16 T. brucei CRK3 (TbCRK3his)

For TbCRK3his purification, BL21 DE3 *E. coli* cells were transformed with plasmid pGL1074 (were grown to 0.6 O.D. at 37°C and induced for protein expression at 19°C overnight using IPTG at 2.5mM following the same procedure used for LmeCRK3his.

2.3.17 T. brucei CKS1 (Tbp12^{CKS1}his)

For Tbp12^{CKS1}his (CKS1 yeast homologue) purification, BL21 DE3 *E. coli* cells were transformed with plasmid pGL976 (were grown to 0.6 O.D. at 37°C and induced for protein expression at 19°C over night using IPTG at 2.5mM following the same procedure used for LmeCRK3his.

2.4 Protein quantification

Protein elution was quantified using PIERCE BCA™ Protein Assay Kit. Standard protein BSA was used to plot concentration curves according to manufacture's instructions for all the proteins quantified.

2.5 Protein kinase assays

Protein kinase assays were performed using 50mM MOPS pH 7.2, 20mM $MgCl_2$, 10mM EGTA, 2mM DTT, 4µM ATP, γ-P^{32}ATP and 2.5µg histone H1 as substrate. Reactions are incubated at 30°C for 30 min. Final volume of each reaction was 20 µl and at the end of the 30 min incubation 20 µl of 2 x Laemmli protein loading buffer was added to stop the reaction, samples then were incubated at 100°C for 5 min and loaded on 12% acrylamide gel. The gel was dried and exposed to KODAK sensitive film overnight. Alternatively to histone H1, 2.5 µg of β-casein or Myelin Basic Protein (MBP) was used.

Table 2.2 List of constructs generated for protein expression

Plasmid number	Gene	Solubility	Expression level
pGL1218	LmCYC6	Soluble	Low levels of expression
pGL1334	LmCYCA	Soluble	Low levels of expression
pGL1335	LmCYC7	Insoluble	Low levels of expression
pGL1336	LmCYC9	Soluble	High levels of expression
pGL1337	LmCYC10	Insoluble	Low levels of expression
pGL1350	LmCYC3	Insoluble	Low levels of expression
pGL1338	LmCRK1	Soluble	High levels of expression
pGL1339	LmCRK2	Soluble	High levels of expression
pGL1340	LmCRK3	Soluble	High levels of expression
pGL1341	LmCRK6	Soluble	Low levels of expression
pGL1342	LmCRK8	Soluble	Low levels of expression
pGL1349	LmCRK7	Soluble	High levels of expression

2.6 Binding assay

To assess whether CRK3 can bind CYCA *in vitro,* a non his-tag LmCRK3 was subcloned into expression vector pET21a (pGL1071). This construction was used to express LmCRK3 in *E. coli* BL21 competent cells and the cell extract was used in a binding assay. A lysate was made at 50 ml of an overnight induced cell culture, incubated with lysozyme and sonicated so that this extract could be used in the binding assay using an affinity column with LmeCYCAhis. BL21 DE3 *E. coli* cells were transformed with plasmid pGL630 and were grown to 0.6 O.D. at 37°C and induced for protein expression at 19°C over night using IPTG at 5mM. Cells were centrifuged and resuspended in PBS 7.4 incubated with DNAse-I (0.1mg/ml) and Lysozyme (0.1mg/ml) for 60 min on ice and sonicated 5 x 15sec (1sec on/1sec off). The lysate was centrifuged 15000g for 20 min. The supernatant was incubated with 200 µl of Ni-NTA agarose bead for 5 min at room temperature and centrifuged for 5 min at 2100g. This column of Ni-NTA + CYCAhis was washed 2 x with PBS 7.4 and incubated with the soluble bacteria lysate containing non taggedCRK3 for 30 min, mixing at room temperature to permit the binding of the two proteins. The beads were then centrifuged at 1000g for 5 min. The column was washed 2X with PBS 7.4 and eluted in 100 µl fractions with phosphate buffer consisting of 100mM NaPi 7.4, 10mM NaCl and 0.5M imidazole (pH 8.0). 10 µl of elution fraction was mixed to 10 µl Laemmli protein buffer 2 x and the total volume of 20 µl was loaded on a 12% SDS-PAGE gel. The proteins on the gel were transferred to a PVDF membrane and a westem blot was performed using α-CRK3 antibodies diluted 1:2000 in PBS. The α-rabbit peroxidase conjugate was used at 1:5000 dilution. The membrane was developed using PIERCE Quimioluminescense kit.

2.7 Transfection of *Leishmania*

Leishmania was grown to middle log-phase. Cells were pelletted at 1300g for 10 min and washed in half of the original volume of cytomix electroporation buffer (120mM KCl, 0.15mM $CaCl_2$, 10mM K_2HPO_4, 25mM HEPES, 2mM EDTA and 2mM $MgCl_2$; pH 7.6) (Robinson and Beverley, 2003). The cells were pelletted again at 1300g for 10 min. The pellet was resuspended in cytomix buffer to a final concentration of 2 x 10^8 ml^{-1}. 10 µg of DNA solution was mixed with 500 µl of cell suspension kept on ice and electroporated twice at 25 µF, 1500 volts (37.5 kV cm^{-1}) pausing 10 sec between pulses. The electroporated cuvettes were then stored on ice for 10 min. The cells were placed in a flask with medium (Homem + 20% FCS) and the flask was incubated overnight at 25°C on its side to increase aeration of the medium. The appropriate antibiotic for selection was added in the following day. For selection of transfected cell lines, the following concentrations of drugs were used: 50µgml^{-1} neomycin, 10µgml^{-1} puromycin, 50µgml^{-1} hygromycin and 10µgml^{-1} blastocydin.

2.8 Preparation of stabilate

DMSO (as a cryopreservative) was used to a final concentration of 5%. 0.5 ml of *Leishmania* (log-phase) was mixed with 0.5 ml of pre-chilled Homem with 20 % FCS and 10% DMSO per cryovial. The tubes were stored overnight at -20°C, transferred to -80°C where were left overnight and then transferred to the liquid nitrogen storage tanks for long term storage.

2.9 Silver staining for acrylamide protein gels

Milli-Q water was used for all steps. The gels were soaked in water as a primary wash to remove running buffer residues. After the gels were fixed with 50% methanol, 5% acetic

acid, 45% water for 20 min under gentle agitation, they were washed in 50% methanol in water. After this wash, gels were washed with water only for 10 min (gels could be left overnight at this stage). A solution containing 0.02% sodium thiosulphate in water was used to sensitize the gels for 1 min. The gels were then washed twice in water for 1 min and were stained with 0.1% silver nitrate (ice cold solution) in water for 20 min at 4^{o}C. If silver nitrate precipitation was observed, the solution was replaced with a fresh one during the staining. After staining, gels were washed twice in water for 1 min and were then developed with 0.04% formaldehyde in 2% sodium carbonate. If the developing solution turned yellow, a fresh solution was used immediately. 5% acetic acid was used to stop the reaction and the storage of the gels was possible in 1% acetic acid solution.

2.10 Preparation of Leishmanial cell lysates

100 ml of mid log-phase *Leishmania* promastigotes were centrifuged at 1300 g for 10 min. The cell pellet was washed in 50 ml of sterile PBS and centrifuged again at the aforementioned speed. The washed cells were then resuspended in 1 ml of *Leishmania* lysis buffer (10% glycerol, 50mM MOPS pH 7.2, 100mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100) and a protease inhibitor mixture was added to a final concentration of 2.5mM 1,10 phenanthroline, 100µgml^{-1} Leupeptin, 5µgml^{-1} Pepstatin A, 500µgml^{-1} Pefabloc SC, 0.5mM EGTA and 0.5mM EDTA. After the addition of protease inhibitors, the cells were vortexed, left on ice for 30 min and centrifuged at 100,000g at 4^{o}C for 45 min. The supernatant (S-100) contained the soluble protein lysate of *Leishmania*, which was used for western blot analysis and protein purification.

Table 2.3 Summary of protein expression conditions

Protein/tag	Plasmid	*E. coli*	Temperature of expression	IPTG concentration
LmeCRK3his	**pGL751**	**BL21 DE3 pLYS**	**19^{o}C**	**150µM**
LmeCYCAhis	**pGL630**	**BL21 DE3 pLYS**	**19^{o}C**	**5mM**
Civ1-GST	**pGL716**	**BL21 DE3 pLYS**	**21^{o}C**	**0.4mM**
LmCYC3his	**pGL1350**	**BL21 DE3 pLYS**	**19^{o}C**	**5mM**
LmCYC7his	**pGL1335**	**BL21 DE3 pLYS**	**19^{o}C**	**5mM**
LmCYC9his	**pGL1336**	**BL21 DE3 pLYS**	**19^{o}C**	**1mM**
LmCYC10his	**pGL1337**	**BL21 DE3 pLYS**	**19^{o}C**	**5mM**
LmCRK1his	**pGL1338**	**BL21 DE3 pLYS**	**19^{o}C**	**1mM**
LmCRK2his	**pGL1339**	**BL21 DE3 pLYS**	**19^{o}C**	**1mM**
LmCRK3his	**pGL1340**	**BL21 DE3 pLYS**	**19^{o}C**	**1mM**
LmCRK4his	**pGL1616**	**BL21 DE3 pLYS**	**19^{o}C**	**1mM**
LmCRK6his	**pGL1341**	**BL21 DE3 pLYS**	**19^{o}C**	**1mM**
LmCRK7his	**pGL1349**	**BL21 DE3 pLYS**	**19^{o}C**	**1mM**
LmCRK8his	**pGL1342**	**BL21 DE3 pLYS**	**19^{o}C**	**1mM**
TbCRK3his	**pGL1074**	**BL21 DE3 pLYS**	**19^{o}C**	**2.5mM**
Tbp12^{CKS1}his	**pGL976**	**BL21 DE3 pLYS**	**19^{o}C**	**2.5mM**

2.11 Western blot

Protein samples were transferred from the gel to nitrocellulose membrane by using BIORAD semi-dry blotting system for 30 min. After transferring, the membrane was blocked for 1 hour in PBS/Tween 20 (0.1%)/milk 3.5% and washed 5 times for 5 min with PBS/Tween 20 (0.1%) between and after the antibodies incubation. After washing, the first antibody was diluted at the appropriate concentration in PBS/Tween 20 (0.1%)/milk 3.5% and incubated with the membrane mixing gently for 1 hour. The membrane was then washed again 5 times for 5 min with PBS/Tween 20 (0.1%). The second antibody was diluted at the appropriate concentration in PBS/Tween 20 (0.1%)/milk 3.5% and incubated with the membrane mixing gently for 1 hour. After washing the membrane 5 times for 5 min with PBS/Tween 20 (0.1%), the membrane was developed with undiluted West-Pico Quimioluminescense kit (Perbio).

For western blots to detect histidine tagged proteins, the following antibody dilutions were used: α-his antibody diluted at 1 in 5,000 as primary antibody and α-mouse IgG diluted at 1 in 5,000 for HRP monoclonal (200 $\mu g ml^{-1}$) as secondary.

For western blots to detect GST tagged proteins, the following antibody dilutions were used: Sigma rabbit α-GST antibody diluted at 1 in 2,000 as primary antibody and α-rabbit HPR conjugated antibody as secondary, diluted at 1 in 5,000.

For western blots to detect CRK3, a specific polyclonal antibody was developed against CRK3 in rabbit and the serum was used diluted at 1 in 500. As a secondary antibody α-rabbit HPR conjugated antibody diluted at 1 in 5,000 was used.

For western blots to detect phosphorylated CRK3, a specific monoclonal antibody (Calbiochem anti-phosphothreonine mouse monoclonal antibody, 14B3) diluted at 1 in

1,000 was used. The second antibody used was α-mouse HRP monoclonal antibody (SIGMA) diluted at 1 in 5,000.

Antibody 13 was developed against *L. mexicana* CYCA unique peptide sequence for western blot. Antibody 13 diluted at 1 in 1,000 was used as a primary antibody and anti-mouse antibody conjugated with HRP diluted at 1 in 5,000 was used as a secondary.

For western blots to detect HA tagged proteins, Roche monoclonal mouse HRP conjugated antibody was used diluted at 1 in 500. Since this antibody was already conjugated to HRP, no secondary antibody was necessary.

2.12 Immunofluorescence Assays

2.12.1 Fixation

Leishmania was washed twice in PBS before fixation in 200 µl of 1% formaldehyde/ PBS for 30 min at room temperature. The cells were then permeabilised by addition of 20 µl of 1% Triton X-100/PBS for 10 min. 20 µl of 1M glycine/ PBS were added to neutralise the free adelhyde bounds resulting for the formaldehyde fixation, in order to diminish any background fluorescence. The cells were incubated a further 10 min. In the meanwhile, glass slides were washed with 70% ethanol and air dried. The treated cells were then pipetted on the clean slides and left to sediment and adhere to the surface until completely dried.

2.12.2 Immunofluorescence

The primary antibody was diluted 1 in 500 in 0.1% Triton X-100, 0.1% BSA and added to the top of the slide to incubate for 1 hour at room temperature or alternatively overnight at 4^{o}C. The cells were then washed 3 times with 1 ml of PBS. The secondary antibody was

diluted at 1 in 1,000 in 0.1% Triton X-100, 0.1% BSA , added to the cells and incubated in the dark for 1 hour at room temperature. The slides were then washed 3 times with PBS to remove the excess of antibody and left drying (but not completely) before adding a mounting solution (2.5% DABCO in 50% glycerol containing 0.5$\mu g ml^{-1}$ of DAPI. The primary antibody used was Roche monoclonal mouse antibody diluted at 1 in 200 and the secondary antibody was α-mouse FITC conjugated diluted at 1 in 1,000.

2.13 Immunopreciptation (IP)

L. major friedlin strain was transfected with pGL1388 and pGL1389 generating cell lines that would overexpress LmCYCA containing N-terminal (stabilate No. 8105) and C-terminal (stabilate No. 8106) HA tag respectively. HA tagged proteins can be purified with Anti-HA 3F10 antibody that recognizes the 9-amino acid sequence YPYDVPDYA, derived from the human influenza hemagglutinin (HA) protein. The antibody recognizes its antigenic determinant even when the HA peptide epitope is introduced into unrelated recombinant proteins by a technique known as "epitope tagging." This antibody is immobilized into an affinity matrix by Roche in an Anti-HA Affinity Matrix.

These cell lines were grown to mid log phase and 50ml of culture was pelletted at 1000g for 10 min at 4°C. The cell pellet was then washed twice in cold PBS and resuspended in 1ml of IP lysis buffer containing protease inhibitors. To this lysis suspension, 50 µl of HA affinity purification matrix (Roche) was added and an overnight incubation at 4°C with agitation was done. The matrix was then washed 3X with 1ml of lysis buffer and resuspended in 50 µl of lysis buffer. 10 µl was loaded in an SDS-PAGE gel, which was used in western blot or silver stained, while 5 µl was used in a kinase assay using H1 as substrate.

The IP procedure described above for LmCYCA was also used for LmCRK6 HA tagged. Transfected cell lines (stabilate No. 7653 and 7654) were obtained using plasmids pGL1394 and pGL1392. For pGL1392 transfected cell lines (7653) a C-terminal HA tag is added to LmCRK6 for overexpression. For pGL1394 transfected cell lines a C-terminal HA tagged LmCRK6 is expressed by integration of the construct in the rDNA locus.

2.17 Cloning of untagged CRKs

L. major CRKs were excised from their plasmids using restriction sites *Bam*HI and *Xho*I. DNA fragments corresponding to the correct sizes were gel purified and ligated into dual expression vector pACYC Duet. This plasmid, conferring Chlorophenicol resistance to the cells, was previously digested with *Bam*HI and *Xho*I and gel purified. pACYC Duet plasmids containing the *L. major* CRKs were named as pGL1338 (LmCRK1), pGL1339 (LmCRK2), pGL1340 (LmCRK3), pGL1616 (LmCRK4), pGL1341 (LmCRK6), pGL1349 (LmCRK7), pGL1342 (LmCRK8) and pGL1072 (TbCRK3).

2.18 Bacterial Co-transformation

pET15b-derived pGLs containing cyclin genes (LmCYCA, LmeCYCA, LmCYC6, LmCYC7, LmCYC9, LmCYC10 and LmCYC11) were transformed into competent BL21 *E. coli* and selected with ampicillin. The cells were co-transformed with a second plasmid (pACYCA Duet, Chlorophenicol resistant) containing the CRK genes to generate a double resistant *E. coli* strain capable of expressing one His-tagged CYC protein and one non-His-tagged CRK. The CRK genes of interest were LmCRK1, LmCRK2, LmCRK3, LmCRK4, LmCRK6, LmCRK7 and LmCRK8. In total, 49 different strains were generated and some of them were used in expression and co-purification assays (Tables 2.3 and 2.4).

Table 2.4 Co-expression of cyclin-kinase pairs in *E. coli*

	pGL1334	**pGL1218**	**pGL1335**	**pGL1336**	**pGL1337**	**pGL1350**	**pGL630**
pGL1338	CYCA his/CRK1	CYC6 his/CRK1	CYC7 his/CRK1	CYC9 his/CRK1	CYC10 his/CRK1	CYC3 his/CRK1	*Mex*CYCA his/CRK1
pGL1339	CYCA his/CRK2	CYC6 his/CRK2	CYC7 his/CRK2	CYC9 his/CRK2	CYC10 his/CRK2	CYC3 his/CRK2	*Mex*CYCA his/CRK2
pGL1340	CYCA his/CRK3	CYC6 his/CRK3	CYC7 his/CRK3	CYC9 his/CRK3	CYC10 his/CRK3	CYC3 his/CRK3	*Mex*CYCA his/CRK3
pGL1616	CYCA his/CRK4	CYC6 his/CRK4	CYC7 his/CRK4	CYC9 his/CRK4	CYC10 his/CRK4	CYC3 his/CRK4	*Mex*CYCA his/CRK4
pGL1341	CYCA his/CRK6	CYC6 his/CRK6	CYC7 his/CRK6	CYC9 his/CRK6	CYC10 his/CRK6	CYC3 his/CRK6	*Mex*CYCA his/CRK6
pGL1349	CYCA his/CRK7	CYC6 his/CRK7	CYC7 his/CRK7	CYC9 his/CRK7	CYC10 his/CRK7	CYC3 his/CRK7	*Mex*CYCA his/CRK7
pGL1342	CYCA his/CRK8	CYC6 his/CRK8	CYC7 his/CRK8	CYC9 his/CRK8	CYC10 his/CRK8	CYC3 his/CRK8	*Mex*CYCA his/CRK8

2.19 Bacterial co-expression and purification

Co-transformed *E. coli* BL21 capable of expressing one his-tagged CYC protein and one non-his-tagged CRK were grown to 0.6 O.D. in the presence of ampicillin and Chlorophenicol. When an O.D. of 0.6 was reached, the culture was transferred to 19^{o}C and induced for protein expression using 1mM IPTG overnight, shaking for better aeration. From the total culture volume of 200 ml, half was used to co-purify cyclin-kinase complexes using the same protocol for purification of CRK3 described above.

2.20 Preparation of CRK3 Aminolink column

To bind CRK3 to the Aminolink resin (PIERCE), 1ml of slurry containing 500µl of resin bed was placed into the protein purification tube (plastic disposable protein purification columns from PIERCE. Storage liquid was left to flow out of the column and 3 ml Coupling Buffer was added to equilibrate the column and left to flow by gravity. The column was closed at the bottom and 2 ml of CRK3 solution at 1mgml^{-1} was added to the column. 200 µl of reducing agent was also added to the protein column mix. The mix was

then incubated at 4°C mixing gently overnight. To block inactive sites of the column after the overnight incubation, 2 ml of quenching buffer and 200 µl of reducing agent were added to the column and incubated mixing at 4°C for 30 min. The column was then washed with 5 ml of washing buffer and finally 3 ml of PBS 0.05% azide was added as a storage buffer.

2.21 Purification of CRK3 interacting proteins from *Leishmania* lysates

The Aminolink column with recombinant CRK3 attached was used to try to purify proteins that interact with CRK3 present in the S-100 *Leishmania* lysate. Immobilized CRK3 was expected co-purify of ligants like cyclins or subunit inhibitors that would be identified by mass-spectrometry. The column was incubated overnight at 4°C with S-100 obtained from 500 ml of cell culture of mid log phase *Leishmania major* cells. The column was then washed in the lysis buffer 2x (3ml) and eluted in 5 ml for ligants with IgG Elution Buffer (0.2 M glycine HCl, pH 3.0). The washes and elutions were concentrated using Vivaspin 4 spin concentrator columns according to manufactures instructions. The washes and elutions were analyzed in SDS-PAGE gels stained with BIORAD sypro ruby staining according to the manufactures instructions.

Table 2.5 List of constructs that were used in the experiments

Plasmid number	Gene
pGL975	**pGEM-T+TbCKS1 PCR product from gDNA (OL1260/OL861) *Nde*I/*Xho*I**
pGL976	**pET15b+TbCKS1 from pGL975**
pGL977	**pGEM-T+TbCRK3 PCR product from gDNA (OL1412/OL427) *Bam*HI/*Sal*I**
pGL978	**pET15b+TbCRK3 *Nde*I/*Bam*HI**
pGL979	**pGEM-T+TbCYC6 box PCR product from gDNA (OL1413/OL1414) *Bam*HI/*Sal*I**
pGL980	**pGEX5X-1+TbCYC6 box *Bam*HI/*Sal*I**
pGL1036	**pET15b+TbCRK3 amplified from pGL798 *Xho*I/*Nde*I**
pGL1071	**pGL751+LmeCRK3 TLM mutant –lacks Thr^{161} residue**
pGL1072	**pET21a+LmeCRK3 *Xho*I-*Nde*I, it expresses a non-tagged version of CRK3**
pGL1073	**pGL900+TbCYC6 3'end on *Nco*I with OL1418 and OL1419/3'flank on *Apa*I with OL1420 and OL1421 (TbCYC6 C-terminal TAP TAG construct)**
pGL1243	**pGEM-T+LmCYCA from gDNA PCR with OL813/OL814 (*Nde*I/*Xho*I)**
pGL1244	**pGEM-T+LmCYCA 5'flank (915bp) from gDNA with OL1750/OL1751 (*Hind*III/*Sal*I) for CYCA KO$^-$ construct**
pGL1245	**pGEM-T and LmCYCA 3'flank (905bp) from gDNA with OL1752/OL1753 (*Sma*I/*Bgl*I) for CYCA KO$^-$ construct**
pGL1246	**pGEM-T+LmCYCA (CDS) (937bp) from gDNA with OL1754/OL1755 (*Nde*I/*Kpn*I) for N-term TAP TAG construct**
pGL1247	**pGEM-T+LmCYCA (CDS) (941bp) from gDNA with OL1754/OL1759 (*Nde*I/*Sma*I) for C-term TAP TAG construct**
pGL1249	**pGL345+3'flank (*Bgl*II/SmaI) and 5'flank (*Hind*III and *Sal*I) of LmCYCA (Ho- construct, Hyg resist)**
pGL1250	**pGL955+LmCYCA from gDNA OL1754/OL1759 *Nde*I/*Sma*I**
pGL1253	**pGEM-T+LmCYC9 from gDNA PCR, OL1770/OL1771 *Xho*I/*Nde*I**
pGL1254	**pGEM-T+LmCRK1 from gDNA PCR, OL1783/OL1784 *Bam*HI/*Nde*I**
pGL1255	**pGEM-T+LmCRK3 from gDNA PCR, OL1787/OL1788 *Bam*I/*Nde*I**
pGL1256	**pGEM-T+LmCRK7 from gDNA PCR, OL1793/OL1794 *Bam*I/*Nde*I**

pGL1265	**pGL345+Flanking regions of CYCA cloned into *Bgl*II/*Sma*I and *Hind*III/*Gal*I (BSD resistant)**
pGL1266	**pGEM-T+LmCRK3 cloned into pGEM-T OL1765/OL1766 *Xho*I/*Nde*I but the gene has two *Xho*I sites**
pGL1267	**pGEM-T+LmCRK7 cloned into pGEM-T L1767/OL1768 *Xho*I/*Nde*I**
pGL1268	**pGEM-T+LmCRK10 cloned into pGEM-T OL1772/OL1773**
pGL1269	**pGEM-T+LmCRK2 cloned into pGEMT OL1785/OL1786 *Bam*HI/*Nde*I**
pGL1270	**pGEM-T+LmCRK4 cloned into pGEMT OL1789/OL1790 *Bam*HI/*Nde*I**
pGL1271	**pGEM-T+LmCRK6 cloned into pGEMT OL1791/OL1792 *Bam*HI/*Nde*I**
pGL1272	**pGEM-T+LmCRK8 cloned into pGEMT OL1795/OL1796 *Bam*HI/*Nde*I**
pGL1334	**pET15b+LmCYCA *Xho*I/*Nde*I**
pGL1335	**pET15b+LmCYC7 *Xho*I/*Nde*I**
pGL1336	**pET15b+LmCYC9 *Xho*I/*Nde*I**
pGL1337	**pET15b+LmCYC10 *Xho*I/*Nde*I**
pGL1338	**pET15b+LmCRK1 XhoI/NdeI**
pGL1339	**pET15b+LmCRK2 XhoI/NdeI**
pGL1340	**pET15b+LmCRK3 XhoI/NdeI**
pGL1341	**pET15b+LmCRK6 XhoI/NdeI**
pGL1342	**pET15b+LmCRK8 XhoI/NdeI**
pGL1349	**pET15b+LmCRK7 from pGL1256 subcloned in NdeI/BamHI C-term His Tag for E. coli expression**
pGL1350	**pET-15b LmCRK3 from pGL1266 subcloned in NdeI C-term His Tag for E. coli expression**
pGL1369	**PCRscript LmeCYCA amplified from pGL630 using OL1935/OL1936, adds C-term HA tag and SmaI/BamHI sites**
pGL1370	**PCRscript LmeCYCA amplified from pGL630 using OL1937/OL1938, adds N-term HA tag and SmaI/BamHI sites**
pGL1371	**PCRscript LmeCYCA amplified from pGL630 using OL1943/OL1944, adds N-term HA tag and XhoI/NotI sites**
pGL1372	**PCRscript LmeCYCA amplified from pGL630 using OL1945/OL1946, adds C-term HA tag and XhoI/NotI sites**
pGL1387	**pET-15b and LmCRK7 into BamHI/NdeI**

pGL1388	**pGL102 and LmeCYCA PCR from pGL630 with OL1938/1937 N-term HA TAG for overexpression**
pGL1389	**pGL102 and LmeCYCA PCR from pGL630 with OL1935/1936 C-term HA TAG for overexpression**
pGL1392	**pGL102 LmCRK6 C-term HA TAG PCR from pGL1341 into SmaI/BamHI OL1939/1940**
pGL1393	**pGL631 LmCRK6 N-term HA TAG PCR from pGL1341 into SmaI/BamHI OL1947/1948**
pGL1394	**pGL631 LmCRK6 C-term HA TAG PCR from pGL1341 into XhoI/NotI OL1949/1950**
pGL1436	**PCRscript C-term HA TAG of LmCYCA OL1935/1936 Episomal (XmaI/BamHI)**
pGL1437	**PCRscript N-term HA TAG of LmCYCA OL1937/1938 Episomal (XmaI/BamHI)**
pGL1438	**PCRscript N-term HA TAG of LmCYCA OL1943/1944 Intergration (XhoI/NotI)**
pGL1439	**PCRscript C-term HA TAG of LmCYCA OL1945/1946 Intergration (XhoI/NotI)**
pGL1483	**pGL631 C-term CYC6 HA TAG cloned into XhoI/NotI, OL2154/2155**
pGL1484	**pGL631 N-term CYC6 HA TAG cloned into XhoI/NotI, OL2156/2157**
pGL1536	**pET21a+ TbCRK3 non-tagged NdeI/BamHI**
pGL1558	**pGL1249 Inserted PUR res gene from pGL236 into BamH/SpeI LmCYCA KO- construct**
pGL1616	**pET15b LmCRK4 into NdeI/BamHI of pET15b**
pGL1621	**PACYC Duet +LmCRK1 from pGL1338 digested with NdeI/BamHI (No tag is expressed)**
pGL1622	**PACYC Duet +LmCRK2 from pGL1339 digested with NdeI/BamHI (No tag is expressed)**
pGL1623	**PACYC Duet +LmCRK3 from pGL1340 digested with NdeI/BamHI (No tag is expressed)**
pGL1624	**PACYC Duet +TbCRK3 from pGL1036 digested with NdeI/BamHI (No tag is expressed)**
pGL1625	**PACYC Duet +LmCRK4 from pGL1270 digested with NdeI/BamHI (No tag is expressed)**
pGL1626	**PACYC Duet +LmCRK6 from pGL1341 digested with NdeI/BamHI**

	(No tag is expressed)
pGL1627	**PACYC Duet +LmCRK8 from pGL1342 digested with NdeI/BamHI (No tag is expressed)**

Chapter 3

Analysis of the cyclin dependent kinase CRK3 and its interaction with CYCA *In vitro*

3.1 Introduction

CRK3 is believed to be the functional cdc2 homologue from *L. mexicana* since it shares a number of attributes with cdc2 from other eukaryotic species: it binds the *S. pombe* protein $p13^{suc1}$ and its *L. mexicana* homologue $p12^{Cks1}$; it phosphorylates the cdc2 substrate histone H1, and its activity correlates with the division status of the parasite, being active in the proliferative life cycle stages (promastigote and amastigote) and inactive in the cell cycle-arrested metacyclic stage (Mottram *et al.*, 1996a;Grant *et al.*, 1998). The predicted protein encoded by the leishmanial CRK3 gene shows the greatest degree of homology to the cdc2 family of serine/threonine protein kinases. CRK3 has an unusual 19-amino acid N-terminal extension when compared with human cdc2, which is not highly conserved in sequence when compared to other trypanosomatid CRK3s and has only one conserved residue, an arginine at position 10 (Hassan *et al.*, 2001). The leishmanial CRK3 contains all the domains and residues characteristic of the serine/threonine protein kinase family. In addition, CRK3 also contains the conserved residues and domains which are important for the regulation of cdc2 activity. This includes equivalent residues to human cdc2 at Thr-14 and Tyr-15, in the ATP-binding domain, and Thr-161. These three residues are highly conserved both within the CDK family and between species (Hassan *et al.*, 2001). This implies that CRK3 activity may be controlled by similar post-translational mechanisms as exist in other eukaryotes (through a regulating kinase, wee1 and phosphatase, cdc25). In addition to that there is the possibility of regulation through conserved threonine residue Thr-161 that is conserved in the trypanosomatid CRK3 at position Thr-178.

Phosphorylation of the conserved threonine residue (T161 in human CDK1 and T160 CDK2) is performed by a separate protein kinase known as the CDK-activating kinase (CAK). In mammalian cells the activating kinase is another cyclin dependent kinase, CDK7/CyclinH associated with a third protein called MAT-1 (Fisher *et al.*, 1995). In yeast cells this activation is performed by a single protein kinase (CAK or Civ1) (Desai *et al.*,

1995). CAK complex phosphorylated a conserved threonine residue to activated CDKs which play an important role in cell cycle control.

The 16-amino acid "PSTAIRE" sequence in cyclin dependent kinases, is a conserved domain involved in the recognition and binding of the cyclin partner (Pines and Hunter, 1989;Jeffrey et al., 1995). The corresponding domain in the leishmanial CRK3 has six substitutions in comparison with Human cdc2 homologue and is highly conserved in other trypanosome CRK3s. The presence of this domain in the leishmanial kinase suggests that cyclin binding may play an important regulatory role.

Cyclin genes have initially being identified from *T. brucei* based on biochemical and yeast complementation assays (Affranchino et al., 1993). Two other genes CYC2 and CYC3 were also described based on functional complementation of a *S.cerevisiae* G1 cyclin mutant (van Hellemond *et al.*, 2000). Although the first described cyclin was later found to be a cyclin (Hammarton et al., 2000) more evidence and data were added about this family of protein in kinetoplastids (van Hellemond *et al.*, 2000;Banerjee et al., 2003;Hammarton et al., 2003b;da Cunha et al., 2005) .

CYCA from *L. mexicana* is a cyclin gene with no homologues in other trypanosomatids and as such, appears to be unique to *Leishmania* species genomes. In addition to that, CYCA from *Leishmania* is more similar to mitotic cyclins by sequence homology to any other known cyclin from vertebrates or yeast and it possesses all features of a mitotic cyclin. Mitotic cyclins like cyclin B, are the key regulatory proteins controlling mitosis in all eukaryotes, where it binds cydin-dependent kinase, cdk1, forming a complex which initiates the mitotic program through phosphorylation of select proteins. It also regulates the activation, subcellular localization, and substrate specificity of cdk1 (Petri et al., 2007). Previous studies have shown that *CRK3* encodes a 35.6kDa CRK with activity towards histone H1 that is essential for the progression of *L. mexicana* cell cycle (Grant *et al.*,

1998). It has also been shown to have 54% of sequence identity with the human cyclin dependent kinase CDK1 and is almost identical to other *Leishmania* species CRK3. *T. brucei* CRK3 and *L. major* CRK3 have 77.8% identity and therefore are highly conserved among these pathogenic organisms. Furthermore, CRK3 is a single copy gene, and mRNA levels are 5-fold higher in the replicative promastigote life cycle stage than in non-dividing metacyclic or mammalian amastigote forms (Grant *et al.*, 1998;Hassan *et al.*, 2001).

The aim of the present chapter was to investigate the *in vitro* interaction of *L. mexicana* CRK3 and *T. brucei* CRK3 with *L. mexicana* CYCA, as well as the activation of these cyclin:kinase complexes and their activity towards histone H1. Additionally, the role of the phosphorylation at the Thr178 residue in CRK3 from *L. mexicana* and *T. brucei* was assessed using yeast Civ1. Finally, *T. brucei* Cks1 homologue (a cyclin dependent kinase accessory protein) was assessed for its role in the *in vitro* cyclin/kinase complex activity.

3.2 Results

3.2.1 Sequence alignments

A sequence alignment for several trypanosomatid CRK3s, human CDK1 and yeast cdc2 was performed using align X program from Invitrogen Vector NTI package (Figure 3.1). The alignment shows that this protein is highly conserved between *Leishmania* species, with 99.5% identity and only one amino acid difference at position 7. It also show that between the trypanosome species (*T. cruzi* and *T. brucei*), the identity observed is 82.6% while comparison of *Leishmania* or Trypanosome CRK3 with human CDK1 or *S. cereviseae* Cdc2 shows identity ranging from 52% to 54% at matching positions (Table 3.1).

A sequence alignment for several *Leishmania* CYCA homologues is shown in Figure 3.2. The sequence alignment shows that this protein is highly conserved between *Leishmania*

species. The sequences from *L. major, L. donovani, L. infantum* and *L. mexicana* were used in the alignment. Only a few substitutions are observed along the sequences. In a phylogenetic tree that compares the CYCA from *L. major* with mitotic cyclins as well as other cyclins it is shown that CYCA clusters with mitotic cyclins A and B suggesting that CYCA is probably a mitotic cyclin (Figure 3.3).

The alignment for $p12^{CKS1}$ from different organisms can be observed in Figure 3.4. The trypanosomatid $p12^{CKS1}$ proteins are well conserved and share a poorly conserved N-terminal extension not present in the human protein. The yeast protein possesses a 9 amino acid insertion in the core of the protein and a C-terminal extension not present in the trypanosomatid and human orthologues (Figure 3.4).

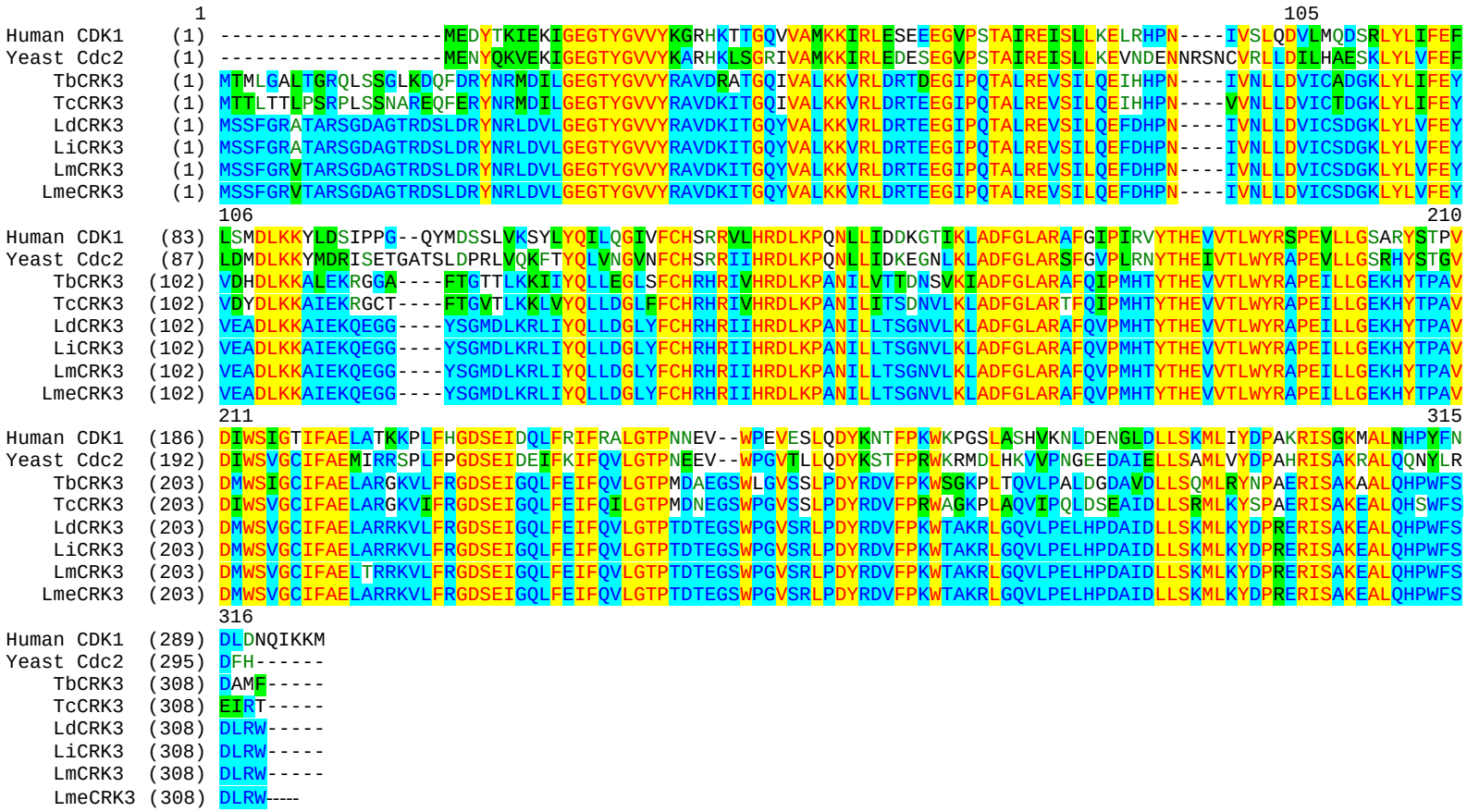


Figure 3.1 Sequence alignments of different species CDK1 homologues. For this alignment human CDK1 from (NP_001777), yeast Cdc2 from *S. pombe* (NP_595629), LiCRK3 from *L. infantum* (XP_001469549), LmCRK3 from *L. major* (LmjF36.0550), LmeCRK3 (CAA04648) from *L. mexicana*, TbCRK3 from *T. brucei* (Tb10.70.2210) and TcCRK3 from *T. cruzi* (Tc00.1047053506583.40) were used. In the alignment the yellow colour represents amino acids residues that are conserved throughout all species, the blue colour represents amino acids residues that are conserved throughout most of the species analysed but not in all of them. These proteins have 38.6% identity among each other. Finally, the amino acid residues that are poorly conserved throughout all the species are represented in green.

Table 3.1 Sequence identity between CRK3 from different species and human CDK1/yeast Cdc2. The comparison shows that LmCRK3 and LmeCRK3 are more similar to yeast Cdc2, with more than 50% sequence identity, than TbCRK3 and TcCRK3. However. TbCRK3 and TcCRK3 are closely related to human CDK1, with more than 49% sequence identity.

	Human CDK1	Yeast Cdc2
LmeCRK3	49.4%	50.2%
LmCRK3	49.1%	50.2%
TbCRK3	49.4%	47.3%
TcCRK3	49.7%	48.6%

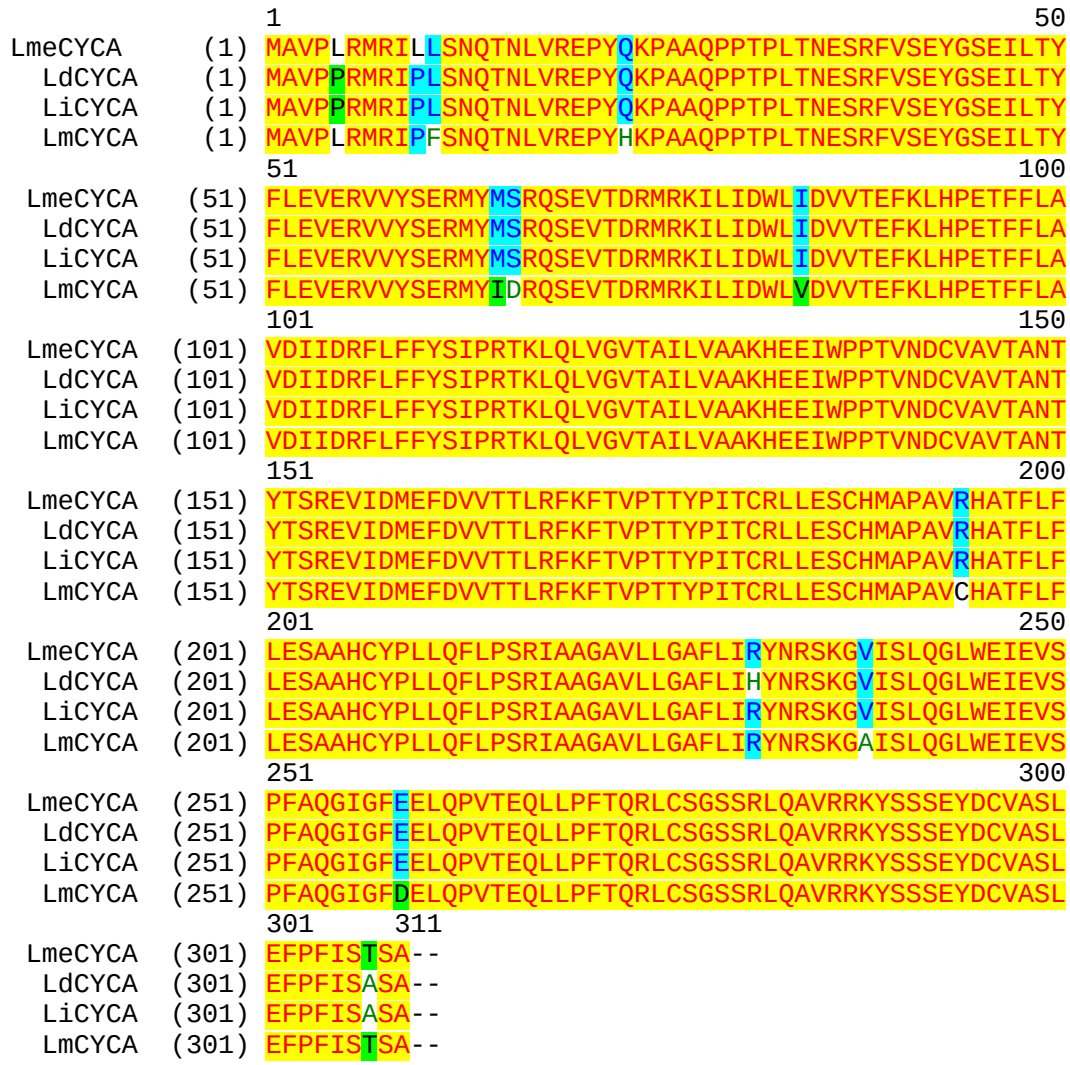


Figure 3.2 Sequence alignment of CYCA from different Leishmania species. LmeCYCA from *L. mexicana* (CAD20131), LdCYCA from *L. donovani* (AAM95631), LiCYCA from *L. infantum* (XP_001466184), LmCYCA from *L. major* (CAJ05300). In the alignment, the yellow colour represents amino acids residues that are conserved throughout all species, the blue colour represents amino acids residues that are conserved throughout most of the species analysed but not in all of them. Finally, the amino acid residues that are poorly conserved throughout all the species are represented in green.

Figure 3.3 A phylogenetic tree of different cyclins. Human Cyclins A1, A2, B1, B2, C, D1, E1, E2 and H were used to be compared with *L. major* CYCA. Sequences from gene bank accession numbers are NM_057749 for human cyclin E2, NM_001238 for human cyclin E1, NM_004701 for human cyclin B2, NM_031966 for human cyclin B1, NM_001237 for human cyclin A2, NM_003914 for human cyclin A1, NM_053056 for human cyclin D1, NM_005190 for human cyclin C, NM_001239 for human cyclin H and LmjF25.1470 for *L. major* CYCA.

Figure 3.4 Sequence alignments of different species CKS1 homologues. For this alignments human CKS1 from (NP_001818), yeast CKS1 from *S. cerevisiae* (YBR135W), LmCKS1 from *L. major* (LmjF32.3790) and TbCKS1 from *T. brucei* (Tb11.01.8085) were used. In the alignment, the yellow colour represents the amino acids residues that are conserved throughout all species, the blue colour represents amino acids residues that are conserved throughout most of the species analysed but not in all of them. Finally, the amino acid residues that are poorly conserved throughout all the species are represented in green.

3.2.2 Purification of recombinant LmeCRK3his

E. coli BL21 DE3 pLYS was transformed with pGL751 (Grant, et al 1998) in order to express LmeCRK3his. This pET28a+ plasmid yielded a high level of expression of soluble LmeCRK3his (Figure 3.5). The cell culture was then used to purify the LmeCRK3his using Ni-NTA agarose metal chelate affinity chromatography (as described in methods). The result from this purification was analyzed (Figure 3.5). Some LmeCRK3his was detected in the insoluble fraction (lane 1), however, when the soluble fraction was analysed most of the expressed LmeCRK3his was detected as a 35 kDa protein (lane 2). This soluble fraction was loaded onto the affinity column and the flow through (FT) fraction was analysed (Lane 3). The affinity column did not remove all of the soluble LmeCRK3his, as some LmeCRK3his was detected in the FT. After the column was washed with sonication buffer containing 20mM imidazole, the bound protein was eluted with 0.5M imidazole, a highly concentrated and relatively contamination free sample of LmeCRK3his was obtained. A western blot using anti-histidine antibody was performed to confirm that the purified protein was indeed LmeCRK3his. A protein around 35kDa, corresponding to the LmeCRK3his was detected (lanes 5-8). The eluted fractions containing purified LmeCRK3his were usually more than 2mg/ml and around 20mg of this protein could be obtained from 1L of cell culture.

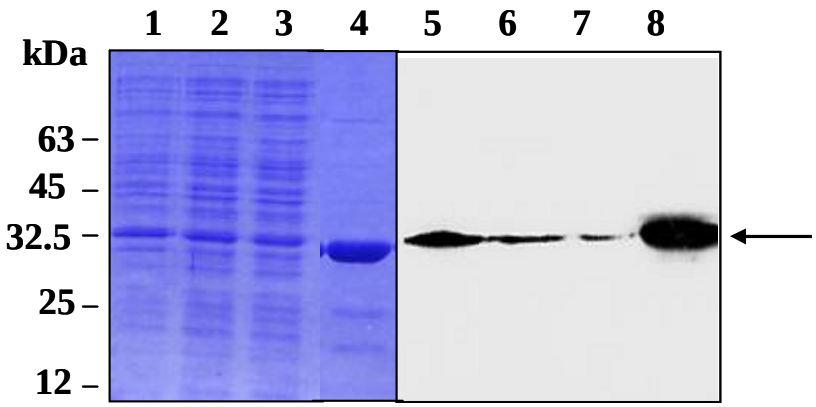


Figure 3.5 *L. mexicana* CRK3his purification and western blot. E. coli BL-21 DE3 pLYS cells were used to induce the expression of LmeCRK3his from pGL751. The purification of the IPTG induced culture was carried out and samples were analyzed on an SDS-PAGE gel stained with Coomassie Blue (Lanes 1-4) and analysed by western blot (Lanes 5-8). Sonicated cell suspension was centrifuged at 20.000 g, pellets were resuspended and analysed as the insoluble fraction (lane1 and 5). The total supernatant was analysed in lane 2 and 6 and was used in the purification, where it was incubated with the Ni-Agarose column. The flow through of this incubation was analysed in lanes 3 and 7. Finally, the Elution of the column can be observed in lanes 4 and 8. Lanes 5-8 correspond to western blot carried out with mouse α-his antibody diluted 1:5,000 as primary antibody and α-mouse HRP conjugated antibody as secondary, diluted 1:5,000. A protein around 35kDa (arrowed) can be observed in the Coomassie and in the western blot, this protein corresponds to LmeCRK3his.

3.2.3 Purification of recombinant LmeCYCAhis

Plasmid pGL630 (Ali, 2002) is based on the pET21a+ vector that adds a 6 histidine tail to the C-terminal end of the protein and allows purification using Qiagen Ni-NTA agarose beads. This vector was used to express and purify recombinant CYCA (Figure 3.6). However, in contrast to LmeCRK3his, LmeCYCAhis from pGL630 did not give a high level of expression. On Coomassie staining of a SDS-PAGE gel a protein around 34kDa was detected weakly in the elution fraction (lane 4). Confirmation that the 34kDa protein was LmeCYCAhis was achieved by western blot (Figure 3.6) using α-his antibody (lanes 5-8). Purified LmeCYCAhis was used to test if recombinant LmeCRK3his could be activated. In the best preparations, purified LmeCYCAhis samples could provide a total protein concentration of 0.2 mg/ml giving around 0.2 mg from 1L of *E. coli* culture. However, the same yield was not reproducible and pGL630 showed a much lower expression levels in subsequent preparations. The elution of soluble LmeCYCAhis from the columns yielded amounts of protein that were not always detectable in Coomasie stained gels; instead, silver stained procedures had to be implemented to actually visualise the presence of CYCA in the gel. A western blot using α-his antibody was again used to confirm the presence of LmeCYCAhis. In addition to the decreased levels of expression of CYCA, much higher levels of contaminating proteins were also observed.

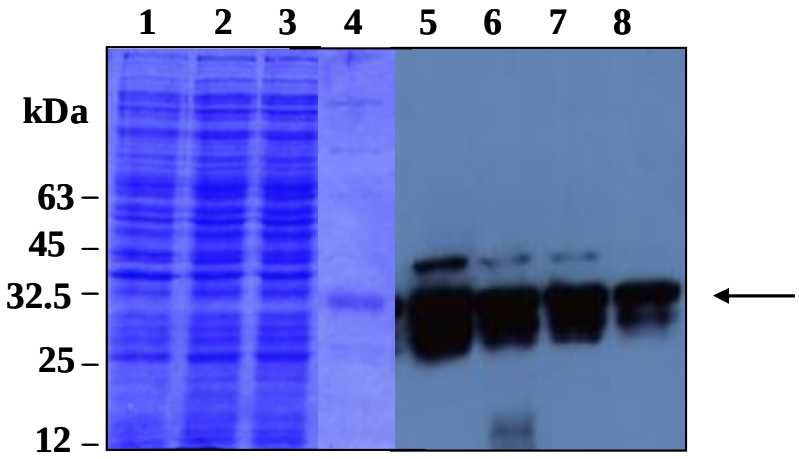


Figure 3.6 LmeCYCAhis purification and western blot. *E. coli* BL-21 DE3 pLYS cells were used to induce the expression of LmeCYCAhis from pGL630. The purification of the IPTG induced culture was carried out and analyzed on SDS-PAGE gel stained with Coomassie Blue (Lanes 1-4) and identification analysed by western blot (Lanes 5-8). The same procedure used to purify LmeCRK3 was used. Sonicated cell suspension was centrifuged at 20.000 g, pellets were resuspended and analysed as the insoluble fraction (lane1 and 5). The total supernatant was analysed in lane 2 and 6 and was used in the purification where it was incubated with the Ni-Agarose column. The flow through of this incubation was analysed in lanes 3 and 7. Finally, the Elution of the column can be observed in lanes 4 and 8. Lanes 5-8 correspond to western blot carried out with mouse α-his antibody diluted 1:5,000 as primary antibody and α-mouse HPR conjugated antibody as secondary, diluted 1:5,000. A protein (arrowed) around 35kDa is identified in the Coomassie and in the western corresponding to LmeCYCAhis.

3.2.4 Expression of recombinant LmeCRK3:

LmeCRK3 was inserted into pET21a to generate pGL1072 (see methods), a plasmid used to transform BL21 DE3 pLys and to express a non tagged LmeCRK3. The soluble fraction of induced *E. coli* culture was used in binding assays using LmeCYCAhis. Cells were grown to an O.D. of 0.7 and induced over night at 19^{O}C with 2mM IPTG. A soluble fraction containing untagged CRK3 was obtained as described for LmeCRK3his (Figure 3.7, lane 2).

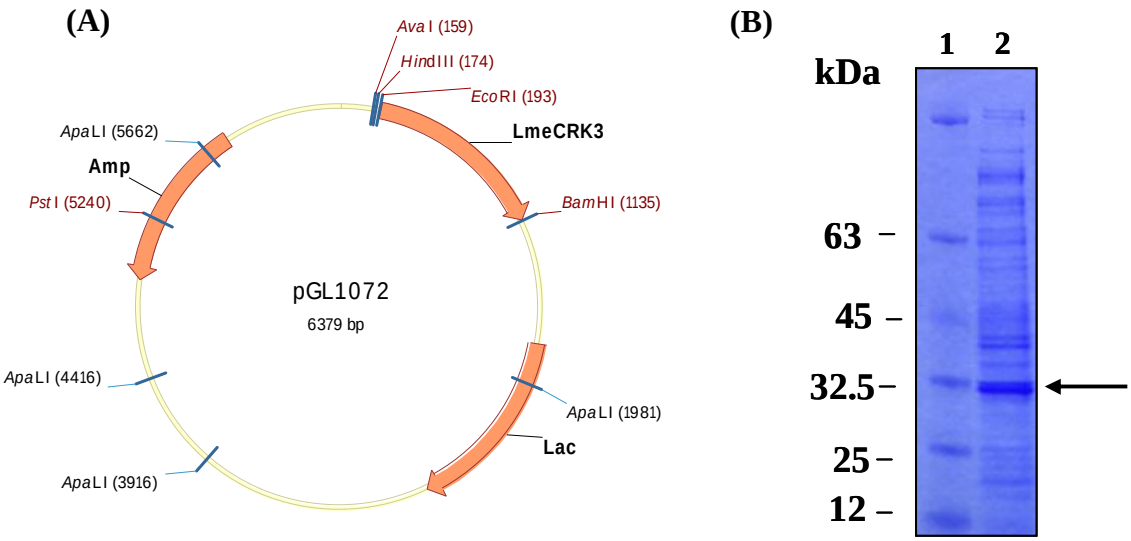


Figure 3.7 (A) A map of plasmid, pGL1072, which is a pET21a derived vector that expresses a non-tagged version of *L. mexicana* CRK3. (B) A Coomassie gel. Lane 1; protein markers lane 2; the expressed *E. coli* soluble lysate obtained after sonication and centrifugation at 20.000g for 20min. The arrow shows LmeCRK3.

3.2.5 Purification of recombinant LmeCRK3his^{T178E}.

LmeCRK3his^{T178E} is a mutant CRK3 from *L. mexicana* derived from pGL751. It was developed by site direct mutagenesis (Ali, 2002) and the threonine 178 residue was replaced by a glutamic acid. The plasmid containing the mutated CRK3 gene was named pGL1071 and was used to express and purify LmeCRK3his^{T178E} (Figure 3.8). As it was derived from pGL751, the levels of expression and purity of LmeCRK3^{T178E}his, as well as the obtained protein concentration, were very similar to that observed in the wild type LmeCRK3his (Figure 3.8).

3.2.6 Purification of recombinant Yeast Civ1protein

A plasmid containing the *Saccharomyces cerevisiae* Civ1 gene (Brown *et al.*, 1999) was obtained from the Endicott group (Oxford University, Biochemistry Department) and was named pGL716. This plasmid expresses a Civ1-GST fusion from pGEX-5X1. Conditions for the expression and purification are described in methods. In BL21 DE3 pLYS, pGL716 provided low levels of expression of the Civ1-GST fusion protein (Figure 3.9). This 66 kDa protein was detected by SDS-PAGE gel stained with Coomassie blue and by western blot with α-GST antibody (Figure 3.9). Although low levels of expression were obtained, the sample was relatively free of contaminants and contained a highly active protein kinase. The combined eluted fractions containing purified Civ1 were usually around 0.5mg/ml.

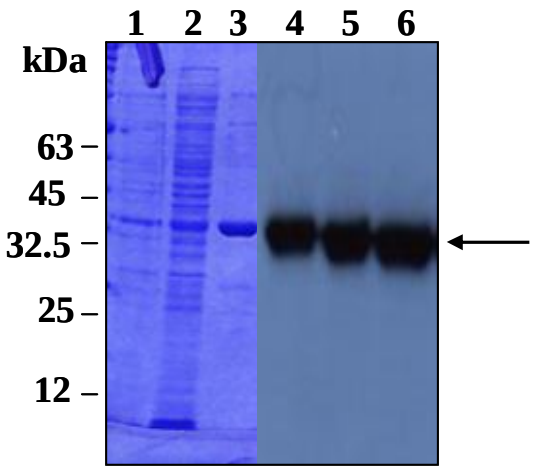


Figure 3.8 Purification and western blot of *L. mexicana* LmeCRK3^{T178E}his. The same procedures used to purify LmeCRK3his were used for LmeCRK3^{T178E}his. The purification of the induced protein was carried out and analyzed on SDS-PAGE gel stained with Coomassie Blue (Lanes 1-3). Lane 1: Insoluble fraction. Lane 2: Soluble fraction. Lane 3: Elution. Lanes 4-6 correspond to Western blot of the same samples carried out with α-his antibody. A protein (arrowed) around 35kDa corresponding to the mutated LmeCRK3his can be observed both in the Coomassie and in the western blot.

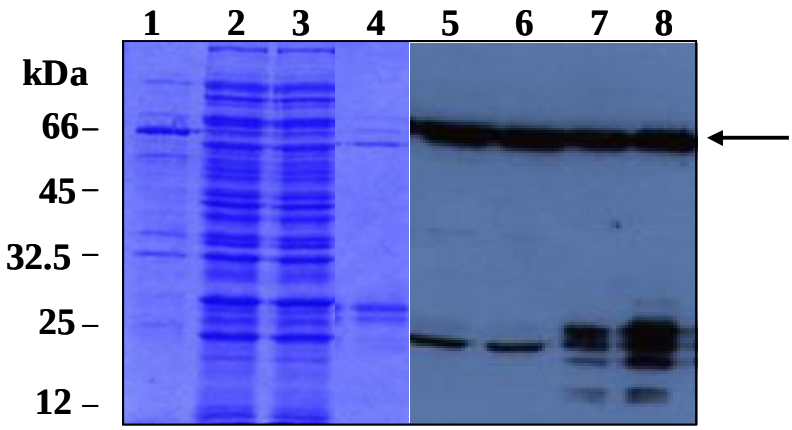


Figure 3.9 Yeast Civ1-GST fusion purification and western blot. *E. coli* BL-21 DE3 pLYS cells were used to induce the expression of yeast Civ1-GST from pGL716. The purification of the IPTG induced culture was carried out and analyzed on SDS-PAGE gel stained with Coomassie Blue (Lanes 1-4) and samples were analysed by western blot (Lanes 5-8). Sonicated cell suspension was centrifuged at 20.000 g, pellets were resuspended and analysed as the insoluble fraction (lane1 and 5). The total supernatant was analysed in lane 2 and 6 and was used in the purification where it was incubated with the Sepharose 4B (Pharmacia) column. The flow through of this incubation was analysed in lanes 3 and 7. Finally, the Elution of the column can be observed in lanes 4 and 8. Lanes 5-8 correspond to Western blot carried out with Sigma rabbit α-GST antibody diluted 1:2.000 as primary antibody and α-rabbit HPR conjugated antibody as secondary, diluted 1:5.000. A protein (arrowed) around 66kDa can be observed in the Comassie and in the western blot, this protein correspond to Civ1-GST.

3.2.7 Purification of recombinant Tbp12^{CKS1}

The gene coding for a *T. brucei* 12 kDa protein corresponding to the yeast CKS1 homologue was cloned in pET15b expression vector generating pGL976. This construct provided high levels of expression of soluble histidine tagged Tbp12^{CKS1} that could be used in kinase assays. A protein of 14 kDa corresponding to the Tbp12^{CKS1}his was observed in the elution fraction indicating its purification (lane 1, Figure 3.10). The western blot with α-histidine antibody confirmed the presence of the purified Tbp12^{CKS1}his, as previously seen on the Coomassie stained gel. Combined eluted fractions containing this protein were normally around 0.1 mg/ml of total protein concentration and around 0.1 mg could be isolated from 1L of cell culture.

3.2.8 Purification of recombinant TbCRK3his:

The *T. brucei* EATRO 795 homologue of LmeCRK3 was cloned from genomic DNA, sequenced for confirmation and subcloned in the expression vector pET15b, generating pGL1036. For *T. brucei* CRK3his purification, BL21 DE3 *E. coli* cells were transformed with plasmid pGL1074 and grown to 0.6 O.D. at 37^{o}C and induced for protein expression at 19^{o}C over night using 2.5mM IPTG. A high level of expression was observed for the *T. brucei* CRK3. A protein around 35 kDa was detected in the elution fraction (Figure 3.11, lane 4) using Coomassie stained SDS-PAGE gels. Confirmation that this was TbCRK3his was achieved by western blot (Figure 3.11, lanes 5-8) using α-his antibody. The protein was detected also in the insoluble, soluble and in the flow through samples (lanes 5-7), but mainly in the elution sample (lane 8). Elutions containing purified CRK3 were usually more than 2mg/ml of total protein concentration. Around 20mg of this protein could be obtained from 1L of cell culture.

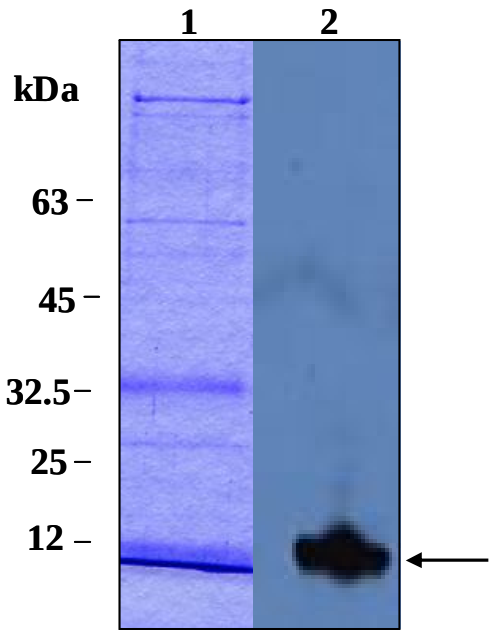


Figure 3.10 *T. brucei* p12^{CKS1}his purification and western blot. *T. brucei* p12^{CKS1}his was purified from E. coli BL21 DE3pLYS containing pGL976 using the same procedure as LmeCRK3his. Western blot using α-his antibody confirmed the size of the purified protein. Lane 1: Coomassie staining of eluted fraction. Lane 2: Western blot of the eluted fraction. A 12kDa protein (arrowed) corresponding to purified Tbp12^{CKS1}his can be observed in both the Coomassie stained gel and the western blot.

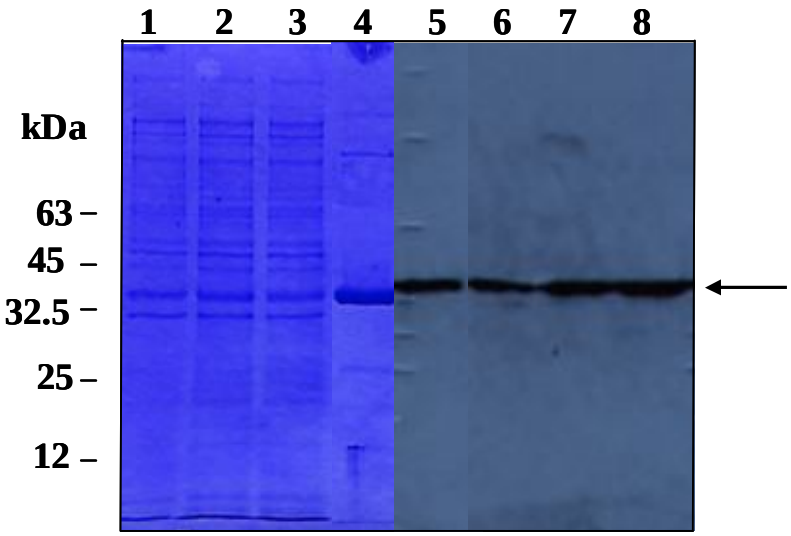


Figure 3.11 Purification and western blot of TbCRK3his. *T. brucei* CRK3his was expressed and purified from *E. coli* BL21 DE3 pLys using pGL1074. The purification of the induced culture was carried out using the same procedure used for LmeCRK3his and analyzed on SDS-PAGE gel stained with Coomassie Blue (Lanes 1-4) and by western blot (Lanes 5-8). Lane 1: Insoluble fraction. Lane 2: Soluble fraction. Lane 3: Flow Through. Lane 4: Elution. Lanes 5-8 correspond to western blot of the same samples carried out with α-his antibody. A protein (arrowed) corresponding to TbCRK3his can be observed in the Coomassie and in the western blot around 35kDa.

3.2.9 In vitro interaction of LmeCYCAhis with LmeCRK3his

To assess if CRK3 and CYCA could interact, a binding assay was designed. Purified LmeCYCAhis bound to the affinity column was used to bind and co-purify a non tagged LmeCRK3 present in a total *E. coli* cell lysate. LmeCRK3 was found to bind LmeCYCAhis immobilized onto the Ni-NTA beads and was co-eluted from the column (Figure 3.12). The binding of LmeCYCAhis to LmeCRK3 was assessed by detection of LmeCRK3 using a polyclonal rabbit antibody against CRK3 (Grant *et al.*, 1998). As shown in Figure 3.12, LmeCRK3 was detected when LmeCYCAhis was present in the column, whereas no LmeCRK3 bound to the column in the absence of LmeCYCAhis. This experiment demonstrates that these two proteins have affinity for each other and bind to form a cyclin kinase complex (Figure 3.12).

3.2.10 Activation of LmeCRK3/LmeCYCA kinase complex

Following the results that showed that CRK3 interacts with CYCA the next step was to test if CYCA would indeed activate CRK3 to provide an active kinase complex. In a previous study, *in vivo* purified CRK3 was show to have kinase activity towards histone H1 (Grant *et al.*, 1998), however the identity of the cyclin partner remained to be determined. Early experiments showed that recombinant monomeric LmeCRK3 had no activity towards histone H1, suggesting that a cyclin was needed to activate the CRK3 kinase from *Leishmania*. The *L. mexicana* CRK3his and CYCAhis purified proteins were tested for kinase activity in an *in vitro* kinase assay using histone H1 as a substrate. LmeCRK3his was shown to exhibit kinase activity dependent on the presence of the LmeCYCAhis (Figure 3.13). LmeCRK3his was activated with LmeCYCAhis, as more kinase activity was observed when more LmeCYCAhis was added to a fixed quantity of LmeCRK3his (lanes 2 to 7, Figure 3.13). No kinase activity was observed in the absence of LmeCYCAhis (lane

1) or in the absence of LmeCRK3his (lane 8). Maximum activity was observed in lane 7, where an approximate 1:1 molar ratio was used.

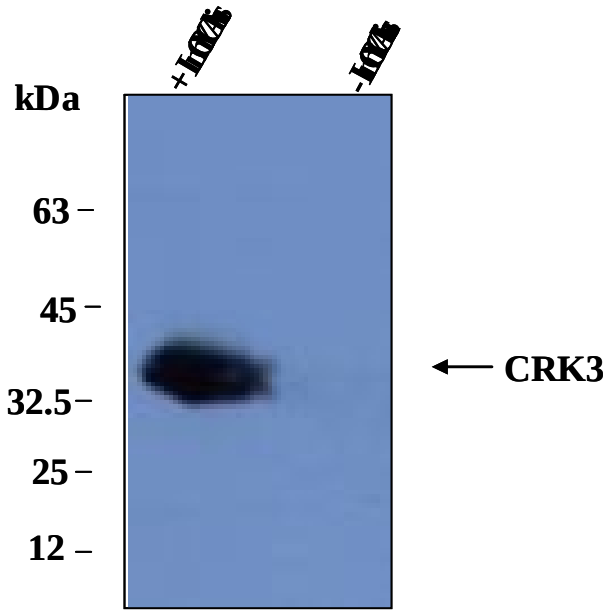


Figure 3.12 LmeCRK3/LmeCYCAhis binding assay. An affinity purification column was made using Ni-agarose beads with bacterially expressed *L. mexicana* CYCAhis. LmeCYCAhis was bound to the Ni-agarose column. This column was used to bind bacterially expressed non-his tagged CRK3 from a soluble *E. coli* lysate. After extensive washing, the elution fraction was tested for the presence of CRK3 by western blot with α-CRK3 antibody. The negative control consisted of Ni-agarose column without LmeCYCAhis bound which was subjected to the same procedure of incubation to an *E. coli* lyasate containing LmeCRK3.

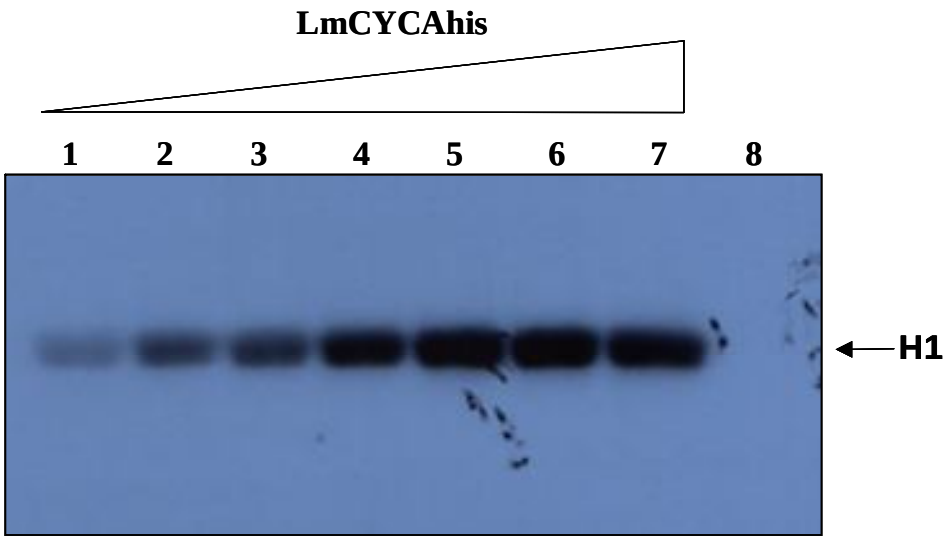


Figure 3.13 Activation of CRK3:CYCA complex. Phosphorylation of histone H1 by *L. mexicana* CRK3:CYCA complex was performed by mixing increasing quantities of LmeCYCAhis (0.5µg on lane 2 to 3µg on lane 8) to a fixed amount of CRK3his (4µg) in an in vitro kinase assay buffer containing 5µg of histone H1 as substrate and γ-ATP labelled with P^{32}. The reaction was incubated at 30°C for 30 min. The reactions were then stopped by adding 2X protein loading Buffer. The samples were then loaded in a 12% SDS-PAGE gel, stained with Coomassie, dried and exposed to a ^{32}P particle emission sensitive film. The gel was exposed over night to a general purpose Kodak film.

3.2.11 Kinase assay using the mutant LmeCRK3^{T178E}his

Following the results showing that CRK3 is able to form a complex with CYCA and that the complex is active in an *in vitro* system, it was possible to use this system to study the role of the residue T178. This residue has been shown to be important for the kinase activity of cyclin dependent kinases in other organisms. Phosphorylation at the threonine residue by a CDK activating kinase increases protein kinase activity of the human CDK2 up to 300 fold (Russo *et al.*, 1996).

Previous results (Ali, 2002), have shown that a substitution at the Thr178 (replacing this threonine for a negatively charged glutamic acid amino acid) does not make monomeric CRK3 active. This was attempted following the suggestions that substitution of the threonine residue for a negatively charged glutamic acid was able to mimic the phosphorylation (Levin and Zoller, 1990) resulting in an active kinase. From these results it was suggested that monomeric CRK3 lacks kinase activity even if it is phosphorylated and that a cyclin partner is essential for activating the kinase. However, it was also possible that the phosphorylation does not have a physiological role in the protein or that the substitution itself resulted in an inactive kinase.

The *in vitro* kinase assay developed for CRK3 and CYCA made it possible to assess the mutated CRK3 in complex with CYCA, as it is known that activation of the CDKs requires both phosphorylation at the Thr178 and binding of the cyclin (Brown *et al.*, 1999). The results presented here show that LmeCYCAhis is not able to activate LmeCRK3^{T178E}his, while it can activate LmeCRK3his (Figure 3.14). This suggests that the mutation completely abolished the kinase activity. Initially, it was thought that the substitution of the threonine residue for a glutamic acid would possibly mimic the negative charge of a phosphorylated protein and therefore fully activates the kinase. However, this was not confirmed in the present study, the substitution of the Thr178 did not further activate the

kinase; instead it caused total loss of activity (Figure 3.14). Increasing amounts of LmeCYCAhis were used to activate wild type LmeCRK3his and LmeCRK3^{T178E}his (Figure 3.14, lanes 1-4). While increasing kinase activity was observed for LmeCRK3his, no activity was detected with LmeCRK3^{T178E}his. A fully activated LmeCRK3his and an inactive kinase corresponding to LmeCRK3^{T178E}his are shown in Figure 3.14. In addition, it can also be observed that LmeCRK3^{T178E}his appears to have a higher autophosphorylation activity than LmeCRK3his (Figure 3.16 lanes 5-8).

3.2.12 Phosphorylation of LmeCRK3his by S. cerevisiae Civ1 in vitro

To test if phosphorylation on the threonine residue of LmeCRK3his by CDK activating kinase would further activate the complex, the *S. cerevisiae* Civ1-GST was purified from *E. coli* as described above.. The yeast Civ1-GST was able to phosphorylate LmeCRK3his, but not LmeCRK3^{T178E}his (Figure 3.14A). These data show that Thr178 is the site of phosphorylation for yeast Civ1-GST. Furthermore, increasing amounts of Civ1 promoted an increase in the amount of phosphorylated LmeCRK3his (Figure 3.15, lanes 2-6), while controls with no Civ1-GST (lane1) or no LmeCRK3his (lane 7) showed no phosphorylation.

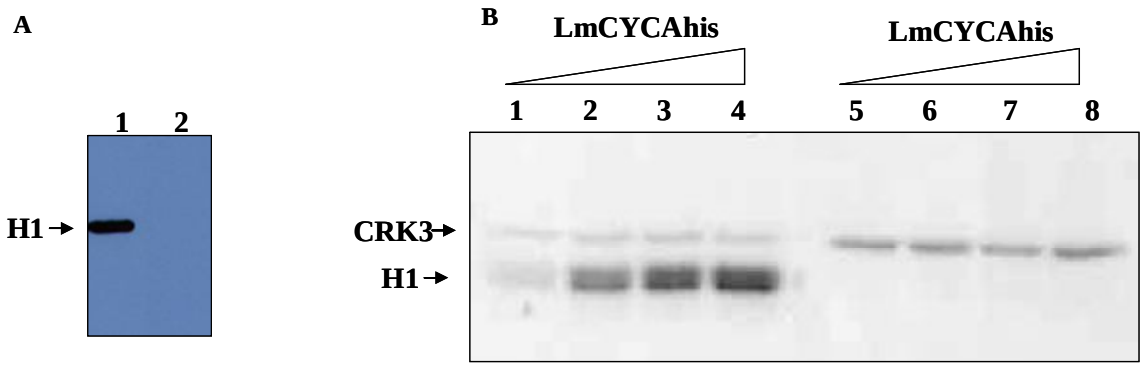


Figure 3.14 LmeCRK3^{T178E}his kinase assay. Panel A, histone H1 kinase activity was assayed using the same procedure described in Figure 3.13. In lane 1 wild type LmeCRK3his was activated with LmeCYCAhis to phosphorylate histone H1. In lane 2 LmeCRK3^{T178E}his in the presence of LmeCYCA shows no kinase activity. Panel B, increasing amounts of CYCA are used to activate LmeCRK3his and LmeCRK3^{T178E}his. CYCA was added at 0.5µg, 1µg, 1.5µg and 2µg. LmCRK3his and LmCRK3^{T178E}his were constant at 3µg in all reactions.

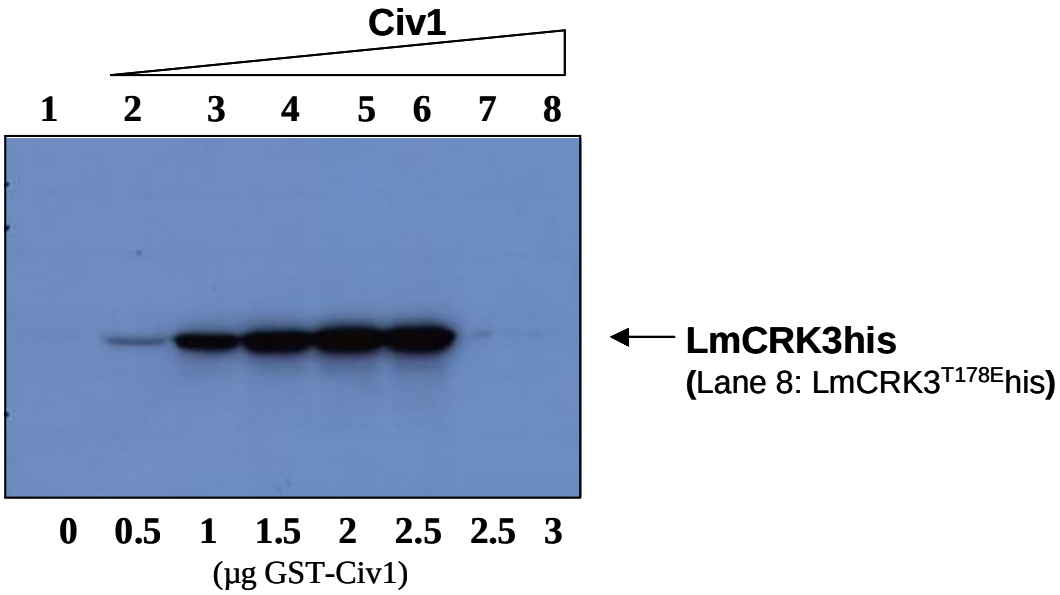


Figure 3.15 Phosphorylation of LmCRK3his by Civ1. LmeCRK3his was used as a substrate for yeast S. cerevisiae Civ1. Lane 1 has no Civ1 and 2µg of CRK3. Lane 2 to lane 6 has increasing amounts of Civ1 from 0.5µg in lane 2 to 2.5µg in lane 6. Lane 7 has no CRK3 and 2.5µg of Civ1. On lane 8, 3µg of LmeCRK3^{T178E}his were added.

3.2.13 Activation of LmeCRK3his by Civ1-GST

In order to assess whether the phosphorylation of Thr178 in LmeCRK3his increased histone H1 kinase activity several experiments were performed. In the first assay (Figure 3.16), LmeCYCAhis and LmeCRK3his were added to two separated sets of reactions. In the first group of reactions (+Civ1), Civ1 was also added, so that LmeCRK3his could be phosphorylated. In the second set (-Civ1), no Civ1 was present and there was no phosphorylation of LmeCRK3his Thr178. The presence of a phosphorylated LmeCRK3 slightly increased the kinase activity (Figure 3.17). The difference in the activity of the phosphorylated LmeCRK3his and the non-phosphorylated LmeCRK3his was quantified using the Pharmacia Typhoon scanner. Phosphorylated LmeCRK3his had its activity increased by 5 times (Figure 3.18).

3.2.14 RINGO kinase activation assay

In order to evaluate the specificity of the activation of LmeCRK3 by LmeCYCA, the ability of RINGO (rapid inducer of G2/M progression in oocytes) to activate LmeCRK3his and LmeCRK3^{T178E}his was tested. RINGO is a protein from *Xenopus* and a potent Cdc2 and Cdk2 kinase activator (Karaiskou et al., 2001) that is not related to the cyclin family and does not require threonine residue phosphorylation for full activation of the kinase. RINGO was not able to activate LmeCRK3his or LmeCRK3^{T178E}his, while it fully activated a *Plasmodium falciparum* cell cycle related protein PfPK5 (Merckx *et al.*, 2003) (Figure 3.19).

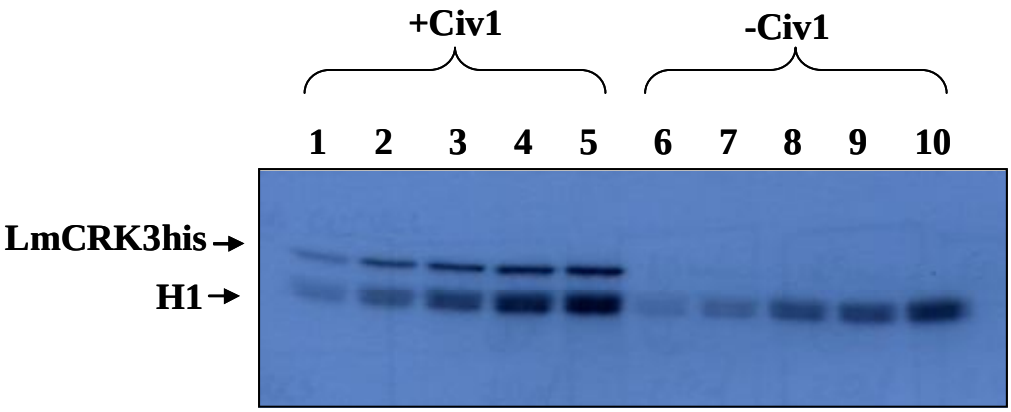


Figure 3.16 Thr178 phosphorylation effect. The procedure for kinase assay was the same as previously described. In order to assess the role of phosphorylation of Thr178 on the activity of CRK3 a time scale was used where the reactions were blocked by adding protein loading buffer after 5 minutes (lanes 1 and 6), 10 minutes (2 and 7), 15 minutes (3 and 8), 20 minutes (4 and 9) and 25 minutes (lanes 5 and 10). LmeCYCAhis (2µg), LmCRK3his (2µg) and Civ1-GST (2µg) were present in constant amounts for all reactions.

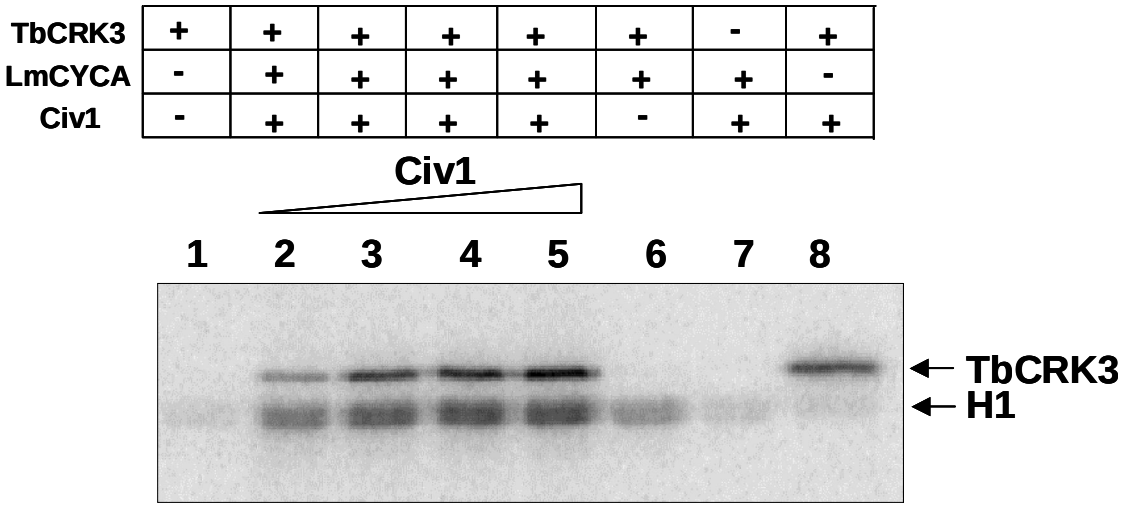


Figure 3.17 T178 phosphorylation effect. A kinase assay using TbCRK3his and LmeCYCAhis was performed to assess the Thr178 phosphorylation effect on the *T. brucei* protein. The kinase assays reactions are as described previously. In lane 1 no Civ1 or LmeCYCA was added and histone H1 was not phosphorylated. From lane 2 to 5 increasing amounts of Civ1 was added to the assay, 0.5µg to 2µg, with constant quantity of TbCRK3his/LmeCYCAhis (2µg). TbCRK3 more intensely phosphorylated in lane 5 and TbCRK3his has its peak activity phosphorylating histone H1. In lane 6 no Civ1 was added and although the histone H1 was phosphorylated, it was less intense than in lane 5, demonstrating the stimulatory effect produced by the activation phosphorylation on Thr178 by Civ1. In lane 8 LmeCYCAhis was not present and Civ1 was still capable of phosphorylating TbCRK3his. As observed for LmeCRK3his, TbCRK3his can be phosphorylated in the absence of a cyclin.

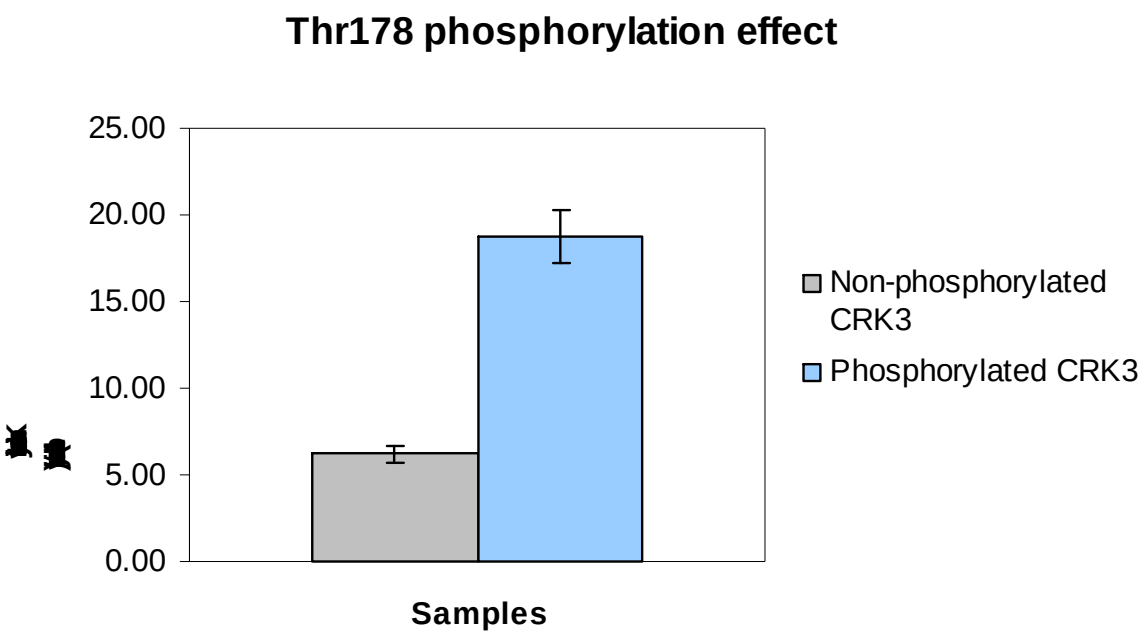


Figure 3.18 The chart shows the difference in kinase activity obtained with the LmeCRK3his combined with LmeCYCAhis in the presence or absence of Civ1-GST. Kinase assays that compared the activity of LmeCRK3his phosphorylated by Civ1-GST with that of the unphosphorylated were analysed with the typhoon phosphoimager (Pharmacia). The band intensities were quantified by the instrument and the mean values were plotted in a bar chart. The error bars represent the standard deviation of the mean.

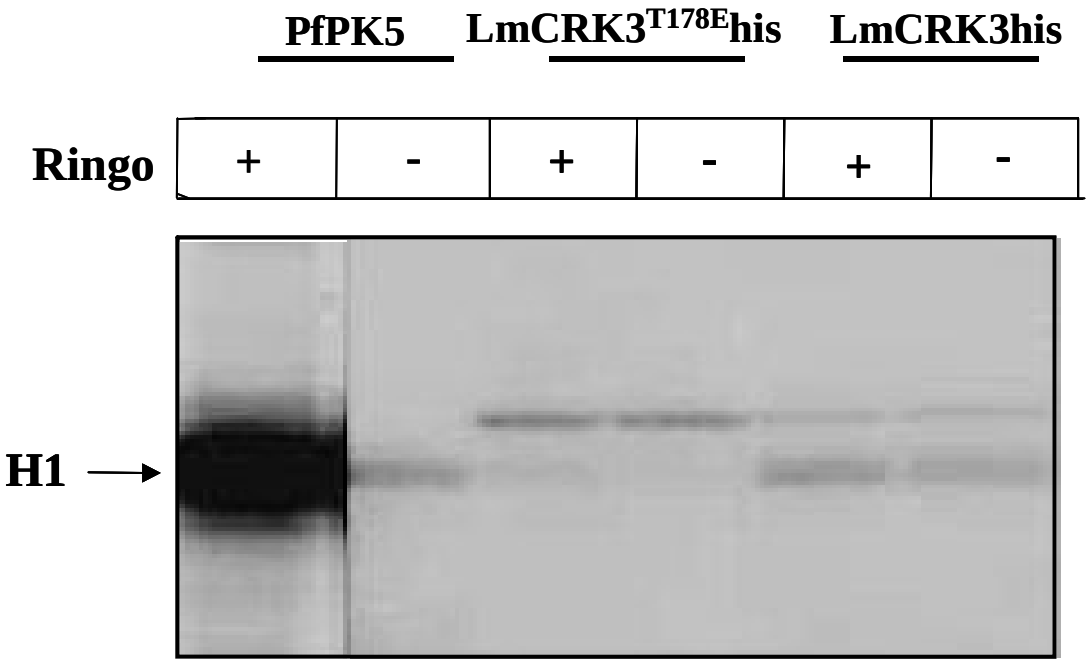


Figure 3.19 RINGO kinase assay. Three proteins (PfPK5, LmeCRK3^{T178E}his and LmeCRK3his) were tested for activation by RINGO protein using as substrate histone H1. PfPK5 was used as a positive control since it is known to be activated by RINGO. Conditions used were the same used for the previous assays. RINGO was added at 0.5µg per reaction, PfPK5 at 0.5µg, LmeCRK3his and LmeCRK3^{T178E}his at 2µg. For each of these proteins two reactions were made, with (+) or without (-) RINGO.

3.3.15 $p12^{CKS1}$ kinase assays

Most cyclin dependent kinases form a tight complex with a small protein known as CKS1. Although its function is not fully understood, it is believed to modulate the kinase substrate affinity (Harper, 2001). Purified $Tbp12^{CKS1}$his (CKS1 homologue) from *T. brucei* was assessed for its effect on LmeCRK3his/LmeCYCAhis and TbCRK3his/LmeCYCAhis kinase activity towards histone H1. $Tbp12^{CKS1}$his had an inhibitory effect on the kinase activity of both LmeCRK3his and TbCRK3his kinases (Figures 3.20 and 3.21).

3.2.16 inhibition of LmeCRK3his/CYCAhis

L. mexicana CRK3 purified from the parasite has been shown to have kinase activity towards histone H1 (Grant *et al.*, 1998). Although it is not known which cyclin activates CRK3 *in vivo* or the Thr178 phosphorylation status, the IC_{50} of the *in vivo* purified CRK3 (Grant *et al*, 2004) could be compared to the recombinant purified LmeCRK3his/LmeCYCAhis. Indirubin-3′-monoxime (Indirubin) (Hoessel *et al.*, 1999) and Flavopiridol (Losiewicz *et al.*, 1994) are two well-studied CDK inhibitors (Grant *et al.*, 2004). IC_{50} values for the *in vitro* complex were similar to those found for the *in vivo* purified CRK3. The concentrations of the compounds used are shown in Table 3.2. The effect of Indirubin and Flavopiridol inhibition can be seen in Figures 3.22 and 3.23. The intensity of the bands were quantified and compared to compound concentration in a Mini Tab plotted graph (Figures 3.24 and 3.25) based on values of band intensities given by Typhoon 860 phosphoimager. IC_{50} values were found to be 25 nM for Flavopiridol and 33 nM for Indirubin.

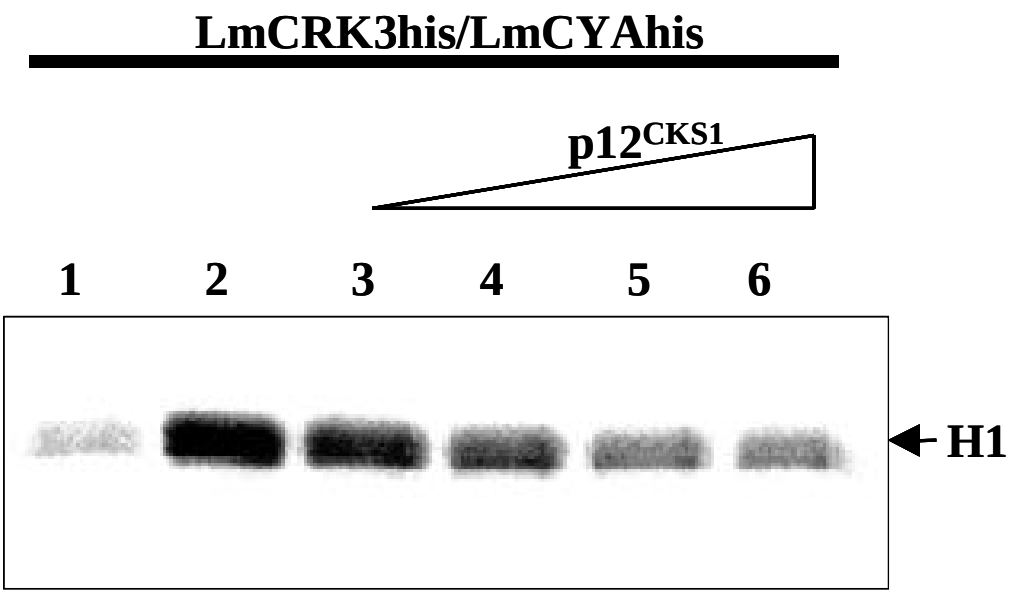


Figure 3.20 $p12^{CKS1}$ assay. $p12^{CKS1}$ protein was assessed for its effect on the kinase activity of the CYCA/CRK3 complex: lane 1, no LmeCYCAhis. All lanes contain 2µg of LmeCRK3his and LmeCYCAhis. $p12^{CKS1}$ was added in increasing amounts (0.5, 1, 1.5 and 2µg) from lane 3 to lane 6 and kinase activity towards histone H1 was assessed.

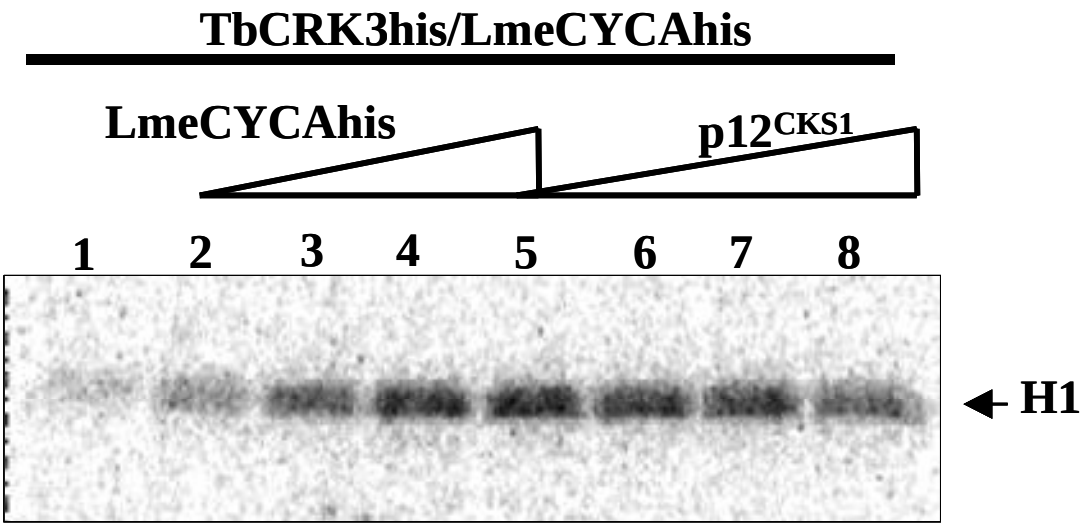


Figure 3.21 $p12^{CKS1}$ assay. In lane 1 no LmeCYCAhis was added. All lanes contained 2µg of TbCRK3his. From lane 2 to lane 5 LmeCYCAhis was present in increasing amounts. The same amount (2µg) of LmeCYCAhis and TbCRK3his were added in lanes 5-8. From lane 5 until lane 8 $p12^{CKS1}$ was added in increasing amounts in the same way as used for LmeCRK3his.

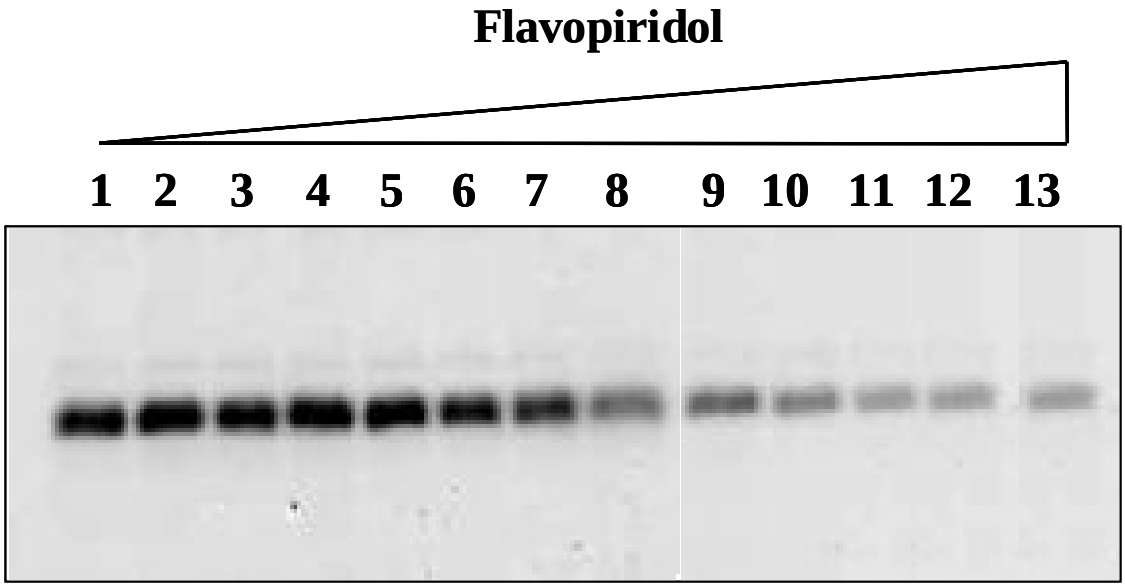


Figure 3.22 Flavopiridol kinase inhibitor activities over LmeCRK3his/LmeCYCAhis complex. Flavopiridol decreased the kinase activity and less phosphorylation of substrate histone H1 was achieved. Flavopiridol concentration increases from lanes 1 to 13. As a result a less intense band was observed. The quantities of the inhibitor added are shown in Table 3.2.

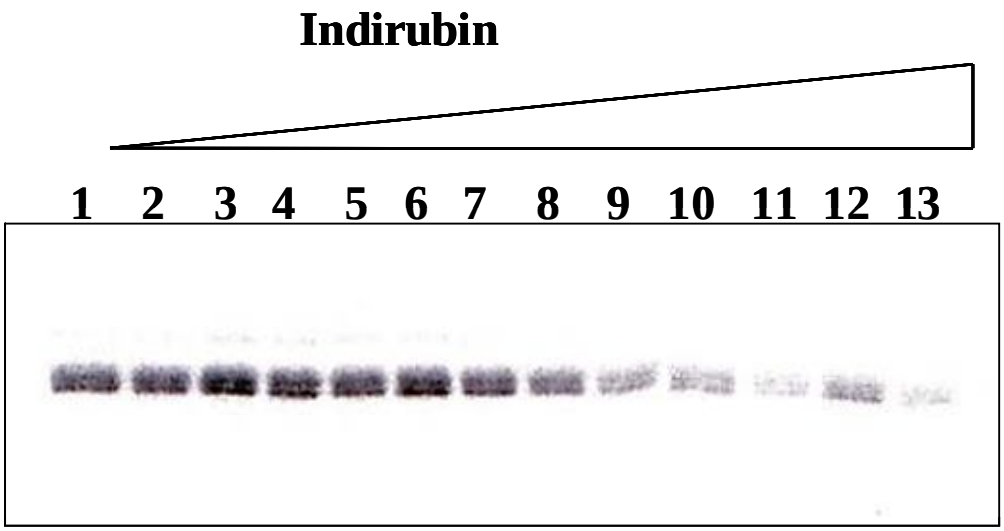


Figure 3.23 Indirubin kinase inhibitor activity over LmeCRK3his/LmeCYCAhis complex. Indirubin decreased the kinase activity and less phosphorylation of substrate histone H1 was achieved. Indirubin concentration increases from lanes 1 to 13. As a result a less intense band was observed. The quantities of the inhibitor added are shown in Table 3.2.

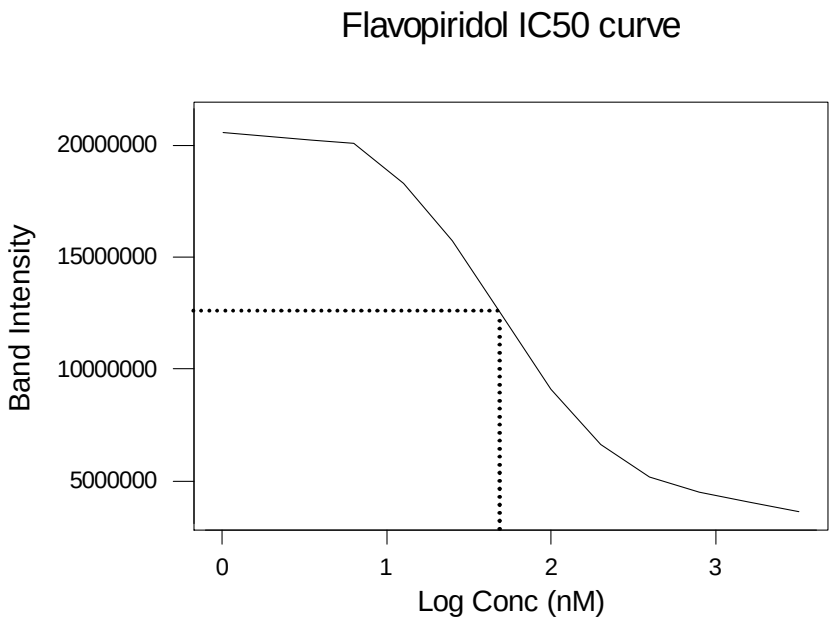


Figure 3.24 Flavopiridol IC^{50} inhibition assay graphic. The gels from the inhibition assays were analysed in the Typhoon scanner and the band intensities were determined. The values were plotted using the program Mat-Lab. Plotted values of Flavopiridol log concentration (x, axis) and band intensity (y, axis) produced the curve in the graphic above that allowed the IC50 value to be determined. IC^{50} for Flavopiridol was 25nM according to Table 3.2.

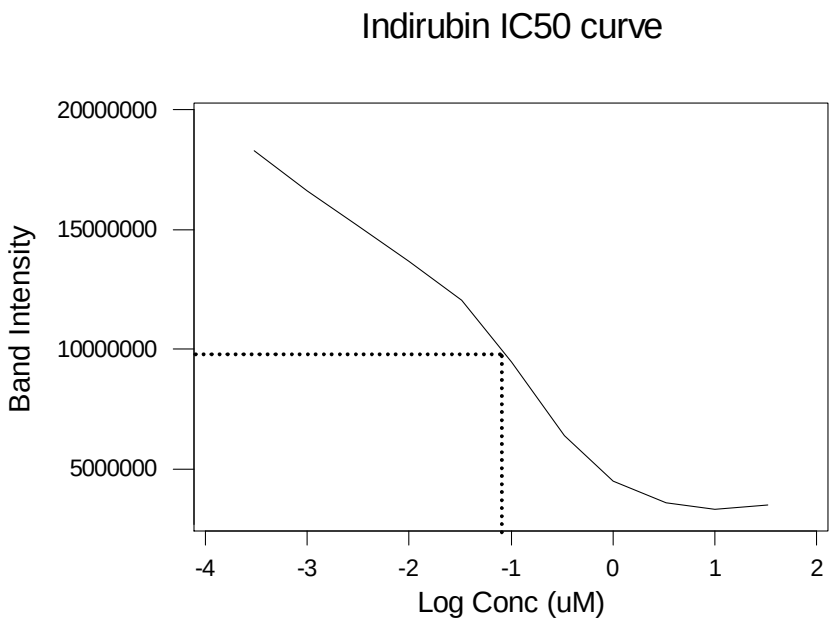


Figure 3.25 Indirubin IC^{50} inhibition assay graphic. The gels from the inhibition assays were analysed in the Typhoon scanner and the band intensities were determined. The values were plotted using the program Mat-Lab. Plotted values of Indirubin log concentration (x, axis) and band intensity (y, axis) produced the curve in the graphic above that allowed the IC50 value to be determined. IC^{50} for Indirubin was 0.033µM according to Table 3.2.

Table 3.2 Indirubin and Flavopiridol inhibition assays. Samples final concentrations in µM (Indirubin) and nM (Flavopiridol) for kinase assay using LmeCRK3:CYCA complex. These concentrations of the inhibitors aforementioned were used to inhibit the complex kinase activity and produce a graph that was used to determine the IC50 values.

Sample number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Indirubin concentration (µM)	0	0.0003	0.001	0.003	0.01	0.033	0.1	0.333	1	3.3	10	33	33
Flavopiridol concentration (nM)	0	3.5	6.25	12.5	25	50	100	200	400	800	1600	3200	3200

3.3 Discussion

The results presented in this chapter have shown that recombinant *Leishmania* CRK3 and CYCA form a complex and that CYCA can activate CRK3 in an *in vitro* system. Although the interaction of CRK3 with CYC6 and CYC2 in *T. brucei* has been demonstrated in an *in vivo* study (van Hellemond *et al.*, 2000;Hammarton *et al.*, 2003a), this is the first time that any recombinant cyclin related kinase from trypanosomatids has been activated in an *in vitro* system. This system, containing the active CRK3, was used in studies that addressed the importance of phosphorylation of Thr178 on the activation site of the structural T-loop characteristic of cyclin-dependent kinases. The protein used to phosphorylate the CRK3 Thr178 in the present study was yeast CAK (also known as Civ1). The homologous protein to Civ1 in *Leishmania* and *Trypanosoma* is still unknown. The active complex was also used in inhibition assays using two kinase inhibitors, Indirubin and Flavopiridol. Finally, the effect of $p12^{Cks1}$ accessory protein was tested and shown to have an inhibitory effect on the CRK3:CYCA activity.

The *Leishmania* and *Trypanosoma* CRK3s were expressed in high levels using the pET vector system in *E. coli*, while the cyclin CYCA was not. The low level of expression of the cyclin may be due to toxicity of the protein to the bacteria or due to DNA structure of the gene in the plasmid, which may impair proper functioning of the plasmid or the bacteria. Only small quantities of protein were observed in Coomassie stained gels, but western blots using α-his monoclonal antibody were able to detect its expression.

The construction of an affinity column where LmeCYCAhis was used to bind non tagged LmeCRK3 demonstrated that these two proteins have strong affinity to each other. There are 10 cyclin genes in *T. brucei* and 11 cyclin genes in *Leishmania* (Naula *et al.*, 2005). CRK3 has been shown to interact *in vivo* with CYC2 (van Hellemond *et al.*, 2000) and CYC6 in *T. brucei* (Hammarton *et al.*, 2003a). *L. donovani* CYCA, also known as CYC1,

was shown to interact with CRK3 (Banerjee *et al.*, 2003;Banerjee *et al.*, 2006). The binding experiment shown in Figure 3.12 confirmed that recombinant *L. mexicana* CYCA was able to bind *L. mexicana* CRK3 and that CYCA may be one of the CRK3 activating partners in *Leishmania*. The purified CYCA and CRK3 were next used for kinase activity.

Activating kinases, such as *L. mexicana and T. brucei* CRK3 with the *L. mexicana* CYCA, was shown to be possible (Figure 3.13). However, the data reported in the current study refers to the activity of proteins expressed and assayed *in vitro*. An active *L. mexicana* CRK3 complex, as well as *L. donovani*, had already been described (Grant *et al.*, 1998;Banerjee *et al.*, 2006), but the *in vivo* activators of *L. mexicana* remained unknown. In *L. donovani* and the immunoblot analysis confirmed that the protein is also expressed during S-phase suggesting its involvement in activities related to DNA replication (Banerjee *et al.*, 2006).

The biochemistry of these complexes was also not accessed. The *in vivo* activator of CRK3 in *L. mexicana* will be accessed in the following chapters. In the present chapter of this study it was shown that kinase activity is achieved independently of the phosphorylation of the Thr178, T-loop activation phosphorylation. However, it is important to point out that it was shown that this residue was free for phosphorylation by Civ1 and therefore was not previously activated by phosphorylation in this site by the *E. coli* lysate. Considering the unphosphorylated status of CRK3, the question that this experiment raised was in relation to the physiological need for phosphorylation of the T178 in the CRK3:CYCA complex. It is known that phosphorylation of the T-loop residue increases kinase activity 100 fold (Brown *et al.*, 1999). The effect of phosphorylation of CRK3 was tested using as controls the unphosphorylated CRK3his, the LmeCRK3his^{T178E} mutant and Civ1 from yeast. The LmeCRK3his^{T178E} was not able to be phosphorylated by Civ1 demonstrating the specificity of the phosphorylation site. The mutant CRK3 was also inactive against histone H1 when complexed with CYCA. Total loss of activity by the

mutant might have been due to conformation failure on the protein's structure caused by synthesis misfolding due to the mutation. The inactivity might have also been due to the inability of the mutant to bind to the cyclin. This could be tested by expressing a non-his tagged CRK3^{T178E} mutant. However as observed in the experiment shown in Figure 3.14, the mutant may autophosphorylate at a higher rate than the wild type CRK3. The autophosphorylation must occur on a Ser or Thr residue since CDKs are not tyrosine kinases. Therefore the autophosphorylation is unlikely to be on the inactivation sites Tyr15 but may occur on the Thr14 or in another unknown site. However, independently of the exact position of this autophosphorylation, it seems to be stimulated in the mutant and might be inactivating the kinase towards H1 substrate. The mutant CRK3 was successfully used to confirm that the phosphorylation of the wild type CRK3 by Civ1 occurs on the Thr178 residue. Based on this finding, the difference in activity between the phosphorylated and nonphosphorylated CRK3 was compared and the role of this important phosphorylation site was assessed.

As it is known from other models like vertebrates and yeast, cyclin binding is one of two major steps in CDK activation; complete activation requires the phosphorylation of the conserved threonine residue (e.g Thr161 in human CDK1) (Gould et al., 1991;Krek and Nigg, 1992;Gu et al., 1992). It has been suggested that affinity of the interaction between CDKs and cyclins may be affected by phosphorylation at the activating threonine. Previous studies have reported that cyclin binding to Cdc2 is inhibited by mutation of the threonine 161, suggesting that phosphorylation at this site stabilizes Cdc2-cyclin complexes (Gould *et al.*, 1991;Ducommun et al., 1991;Norbury et al., 1991). On the other hand, it has also been reported that Cdc2 and cyclin bind with high affinity in the absence of phosphorylation (Desai et al., 1992;Solomon et al., 1992;Atherton-Fessler et al., 1993). For the human CDKs and cyclins, it was shown that CDK1 binds to cyclin B, but not to cyclin A in the absence of Thr161 phosphorylation, while CDK2 binds to both cyclin A and B without requiring Thr161 phosphorylation (Desai *et al.*, 1995). Furthermore, the crystal

structure of the unphosphorylated CDK2-Cyclin A complex revealed that the cyclin induced activation results from conformational changes in the catalytic cleft that realign active site residues and relieve the blockade of the catalytic cleft (Jeffrey *et al.*, 1995). A comparison between the unphosphorylated and phosphorylated CDK2-cyclin A complex revealed that the presence of the phosphate group induces conformational changes in the T-loop and C-terminal lobe of CDK2, as well as new interactions within CDK2 and cyclin A (Russo *et al.*, 1996).

Despite universal conservation of the two-step CDK activation requirement (cyclin binding and CAK phosphorylation), the identity of the CAK has diverged (Fisher, 2005). In metazoans, the only CAK identified to date is the Cdk7/cyclin H/Mat1 complex, which is also a component of the RNA polymerase II general transcription factor IIH. Cdk7 has evolved to recognize two distinct and structurally dissimilar substrates, the T loops of CDKs and the carboxy-terminal domain of the largest subunit of Pol II (Larochelle *et al.*, 2006). Kin28 is the yeast *S. cerevisiae* orthologue of Cdk7, which is a kinase that phosphorylates the carboxy-terminal domain RNA polymerase II complex, but has no CAK activity. T loops of CDKs are instead phosphorylated by Cak1, a single-subunit kinase related only distantly to CDKs (Thuret et al., 1996;Kaldis et al., 1996;Espinoza et al., 1996). Differently, two CAKs are found in the fission yeast *S. pombe*, the Mcs6 complex and the nonessential Csk1. Csk1 is the actual orthologue to the metazoan and budding yeast enzymes (Hermand *et al.*, 1998;Lee *et al.*, 1999;Saiz and Fisher, 2002).

In the present study, phosphorylation of CRK3 by Cv1 from both *T. brucei* and *L. mexicana* was shown to enhance protein kinase activity toward histone H1 by around 5 fold. However, binding of the cyclin to the kinase and the kinase activity on the complex do not appear to be dependent on the phosphorylation of the Thr178. The 5-fold increase in kinase activity observed in the phosphorylated complex was very subtle when compared to the 100 fold increase in the human or yeast complexes (Morgan, 1997). The reason for that

is unknown, but it may be that yeast Civ1 used in the assays did not phosphorylate all of the CRK3 in the reaction. This would have been expected since Civ1 is a heterologous enzyme and the sequence of the phosphorylation site differs in *Leishmania* and *T. brucei* from that of yeast. While in *Leishmania* the sequence is MHTX**T**XXVX, in yeast it is LRNX**T**XXIVT. However, no Civ-like proteins from the *Leishmania* or *Trypanosoma* (Parsons *et al.*, 2005) genomes could be readily identified by sequence homology and as such, yeast Civ1 was used instead. The procedures that are used to phosphorylate the kinase complexes in humans, yeast and CRK3 (the present study) are similar and Civ1 appears to be fully active with CRK3 as a substrate. In addition to that, the protein band corresponding to the phosphorylated CRK3 observed in the autoradiographic films appeared to be as intense as the ones corresponding to the histone H1 substrate suggesting that at least some of CRK3 was indeed phosphorylated by Civ1.

The RINGO protein (Karaiskou *et al.*, 2001) was used as a test to assess the specificity of the interaction between CRK3 and CYCA and the consequent activation of this complex. RINGO (rapid inducer of G2/M progression in oocites) is the putative protein responsible for triggering Cdc2 homologue in *Xenopus* oocytes in the absence of the conserved T-loop phosphorylation. Due to fact that RINGO is not a cyclin and can activate CDKs independently of the Thr phosphorylation, it was also possible that RINGO could activate CRK3 in a similar manner to CYCA. RINGO can directly activate Cdk1 and Cdk2 using bacterially produced proteins (Nebreda, 2006) . Mammalian RINGO/Speedy family members can also bind to and activate Cdk1 and Cdk2, albeit with different efficiencies, but they do not bind to or activate Cdk4 and Cdk6 (Nebreda, 2006). *In vitro* and overexpression experiments suggest that most RINGO/Speedy proteins might bind to and activate Cdk2 more efficiently than Cdk1 (Karaiskou *et al.*, 2001;Porter et al., 2002;Cheng et al., 2005;Dinarina et al., 2005). However, complexes between endogenous RINGO/Speedy proteins and Cdk1 or Cdk2 have yet to be detected. As shown in Figure 3.19 there was no activation of CRK3 by RINGO, whereas the control (a *Plasmodium*

falciparum cell cycle related kinase PfPK5) (Holton *et al.*, 2003) was fully activated by RINGO. This result provides more evidence for the specificity of the activation of CRK3 by CYCA, which is a mitotic cyclin. This does not mean that different cyclins are not able to activate CRK3 as well. This possibility will be addressed in the following chapters.

Human Cks1 and Cks2 were isolated (Richardson *et al.*, 1990b) and it was found that Cks proteins were structurally and functionally conserved throughout eukaryotes and could directly bind to cyclin/CDK complexes. Although it is known that Cks proteins are crucial for CDK function and cell division in yeast, the molecular details of their functions have remained unclear. Recently, Cks1 was reported to be an essential cofactor for SCF^{Skp2} ubiquitin ligase to ubiquitinate $p27^{Kip1}$, whereas Cks2 exhibited no such activity (Harper, 2001;Ganoth et al., 2001;Spruck et al., 2001). $p27^{Kip1}$ is one of the kinase inhibitors regulating G_1-S progression and is degraded via the ubiquitin-proteasome pathway (Pagano *et al.*, 1995;Vlach *et al.*, 1997;Shirane *et al.*, 1999). It was also recently shown that it modulates transcription of the APC/C protein-ubiquitin ligase activator Cdc20 in *S. cerevisiae* (Brizuela et al., 1987;Morris et al., 2003). The cyclin-dependent kinase complexes from several organisms have been purified from cell extracts using their affinity to Cks1 (Brizuela *et al.*, 1987). This protein can be immobilized onto an affinity column and the column can be used to purify the cyclin-dependent kinase complexes from trypanosomatids (Mottram *et al.*, 1996a). In the present study, a recombinant Cks1 homologue from *T. brucei* was cloned expressed, purified and added to the system developed with CRK3:CYCA to analyse the effect on the kinase activity. It was found that *T. brucei* Cks1 had an inhibitory effect on the LmeCRK3his kinase activity toward histone H1 (Figure 3.20 and 3.21). However, the inhibition was less pronounced for the TbCRK3his:LmeCYCAhis complex. The reason for the inhibitory effect has yet to be elucidated. Because Cks1 is believed is responsible for modulating the affinity between the kinase complexes and the substrates (Harper, 2001), it is possible that TbCks1 acts as a modulator in the affinity of the complex towards histone H1.

In order to evaluate the similarity between the active recombinant kinase complex reported here and the *in vivo* purified CRK3 (Grant *et al.*, 2004), the IC_{50} for two different kinase inhibitors (Flavopiridol and Indirubin 5' monoxide) was determined. These kinase inhibitors are known to have different IC_{50} values for different kinases. Flavopiridol IC_{50} for human CDK1 and CDK2 are 500nM and 100nM respectively. Indirubin IC_{50} for CDK1 and CDK2 are 10mM and 2.2-7.5mM (Losiewicz *et al.*, 1994;Carlson et al., 1996;Hoessel *et al.*, 1999). In the present study, the IC_{50} values for the recombinant complex were found to be in a similar range with those from the *in vivo* purified CRK3. *In vivo* purified CRK3his had an IC_{50} of 1350 nM for Indirubin (Grant *et al.*, 2004) while the recombinant LmeCRK3his:LmeCYCAhis complex had an IC_{50} of 33nM for the same compound. For Flavopiridol the *in vivo* complex presented an IC_{50} of 100nM (Hassan *et al.*, 2001) while the recombinant complex was 25nM , suggesting that the former reflects the latter. Given the similar values for the IC_{50} for these 2 kinase inhibitors it could be suggested that CRK3 is activated by CYCA *in vivo* and that phosphorylation on the Thr178 residue may not be essential *in vivo*.

Chapter 4

Cloning, expression and activity of *L. major* CRKs and CYCs

4.1 Introduction

Cyclin-dependent kinases (CDKs) are serine-threonine kinases that play pivotal roles in the control of the eukaryotic cell cycle. In yeast, cell-cycle progression is predominantly regulated by one CDK, p34 cdc2 in *Schizosaccharomyces pombe* or p34 cdc28 in *Saccharomyces cerevisiae* whereas in higher eukaryotes many cdks are involved. To date 13 CDKs and 25 cyclins encoded in the human genome have been described (Morgan, 1997;Pavletich, 1999;Dhavan and Tsai, 2001). Although CDKs were initially studied for their cell cycle functions, it was later observed that some members of the family have roles in different contexts (Knockaert *et al.*, 2002) In humans, for example, four CDKs are involved in the regulation of transcription (Knockaert *et al.*, 2002). CDK7–cyclin-H–MAT1 is a part of the transcription factor TFIIH. Both CDK7–cyclin H and CDK8–cyclin C phosphorylate the large subunit of RNA polymerase II, required for elongation (Fisher, 2005). CDK9–cyclin T is a component of the transcription factor P-TEFb. It is required for the kinase-dependent HIV-1 Tat transactivation (Pumfery *et al.*, 2003). CDK11 is also very important in regulating RNA processing and transcription, in association with the recently described cyclin L (Ania-6) (Hu *et al.*, 2003).

Progression through the G1, S, G2 and M phases of the cell division cycle is regulated by CDKs. In early–mid G1, extracellular signals modulate the activation of CDK4 and CDK6 associated with D-type cyclins (Sherr and Roberts, 2004;Kozar and Sicinski, 2005). The phosphorylation of pRB in G1 by cyclin D/CDK4/6 (and subsequently by CDK2) is believed to be a requisite event in reversing the repressive effects of pRB and de-repressing transcription of a number of genes required for exit from G1 and initiation and completion of S phase. These phosphorylation inactivates pRb and releases the E2F and DP1 transcription factors responsible for essential genes required at G1/S transition (Haberichter *et al.*, 2007). The G1/S phase transition and centrosome duplication is governed by CDK2–cyclin E complex (Cowan and Hyman, 2006). Still during S phase,

CDK2–cyclin A phosphorylates various substrates allowing DNA replication (Kaldis and Aleem, 2005). Around the S/G2 transition, CDK1 associates with cyclin A. Later, CDK1–cyclin B appears and triggers the G2/M transition by phosphorylating several substrates. Phosphorylation of the 'anaphase promoting complex' by CDK1–cyclin B results in transition to anaphase and completion of mitosis (Petri *et al.*, 2007). The ordered action of different CDK–cyclin complexes is dependent on post-translational modifications and intracellular translocations. Complexion of phases are regulated through so-called 'checkpoint' controls (Knockaert *et al.*, 2002).

The fission yeast suc1 gene encodes an essential protein (p13) which interacts with cdc2 and was first identified as a plasmid-borne suppressor of certain temperature-sensitive cdc2 mutants (Hayles *et al.*, 1986). The budding yeast homologue of suc1, CKS1 (cdc2 kinase subunit 1), was identified in a similar manner (Hadwiger *et al.*, 1989). Two human homologues of suc1, ckshs1 and ckshs2 capable of rescuing null mutations of the *Saccharomyces cerevisiae* CKS1 gene (Richardson *et al.*, 1990a). The specificity of binding is high and shown to bind higher eukaryotic cdc2 and CDK2 strongly but not other CDKs (Meyerson *et al.*, 1992). The high affinity with which CKS1 binds to Cdc2 *in vitro* has been exploited in the isolation of cdc2 kinase complexes from a variety of organisms including *Leishmania* (Mottram *et al.*, 1996a).

Unicellular *S. cerevisiae* and *S. pombe* have a single cyclin dependent kinase to control their cell cycle. Other yeast Cdks like PHO85 are also cyclin dependent kinases and do have role in cell cycle progression, however, cell cycle progression occur in its absence suggesting overlapping of functions (Huang *et al.*, 2007). Kin28 and Srb10 are also cyclin activated proteins in yeast that are not essential for cell cycle progression (Espinoza *et al.*, 1998). Also unicellular eukaryotic cells of parasites from the genus *Leishmania* have 11 genes of CRKs (CRKs 1, 2, 3, 4, 6, 7, 8, 9, 10, 11 and 12). CRK5 initially included in the CRK family because of its similarities with Cdc2 was later removed as it showed more

characteristics of MAP kinase than of cyclin dependent kinase. The cyclin family is also large with 11 genes (CYCA, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11) (Naula *et al.*, 2005). The CYC1 protein, initially thought to be a mitotic cyclin (Affranchino *et al.*, 1993) was later found to be more similar to a serine peptidase (Hammarton *et al.*, 2000). CYCA is an exclusive gene of the genus *Leishmania* with no orthologue in trypanosomes. Although it is well known that CDKs associates with its subunit cyclin to be active, only a few cyclin:kinase pairs have being identified in kinetoplastids. In mammalian cells the 10 CDK proteins associates with around 14 different cyclins. These pairs are well established and their function defined (Knockaert *et al.*, 2002). The association of the numerous cyclins with the numerous kinases in kinetoplastids was never assessed in further detail. It is known that CRK3 forms a complex with the CYC6 mitotic cyclin and the association is essential for G2/M phase progression being the functional homologue of CDK1 (Hassan *et al.*, 2001;Hammarton *et al.*, 2003a;Tu *et al.*, 2004;Tu *et al.*, 2005). It is also known that the same CRK3 in complex with CYC2 is essential for G1 progression (Li *et al.*, 2003a;Hammarton *et al.*, 2004).

In yeast, a single protein acts as CDK activating kinase, CAK or Civ1. In humans, another CDK associated to MAT1 is responsible phosphorylation of the Threonine residue at the activation loop (Martinez *et al.*, 1997). In *Leishmania* no CAK/Civ1 or CDK7/MAT/Cyclin H can be identified by sequence homology. Although *Leishmania* CRKs have conserved Threonine at the activation loop, it is still unknown if these protein need phosphorylation for full activation. In chapter 3 of this thesis it was shown that Civ1-GST purified from *E. coli* can phosphorylate LmCRK3 at position Thr178 and that this enhances the kinase activity around 5 times. In this chapter, *Leishmania* CRK1, CRK2, CRK4, CRK6 and CRK7 were cloned, expressed and tested as substrates for Civ1. An analysis of the *Leishmania* cyclin genes was carried out and the CYC9 was found to be the closest related gene product to human cyclin C and cyclin H. *Leishmania* CYC9 was expressed in *E. coli*, purified and used to assess whether it could activate CRK1, CRK2,

CRK3, CRK4, CRK6 and CRK7 in search of the kinase activating kinase complex in *Leishmania*.

Following the successful activation of CRK3 *in vitro* with CYCA and CYC6, a strategy was developed to define cyclin-dependent kinase complexes in *Leishmania*, such as CRK3:CYC6his complex. In the present chapter, the strategy used involved the co-transformation of *E. coli* BL-21 DE3 with two different plasmids, one containing an untagged CRK gene and another containing a histidine tagged cyclin. Co-transformed cells were then used to express and purify possible interacting kinase:cyclin pairs to be tested in kinase assays. The kinase genes used were CRK1-4 and CRK6-8 from *L. major* which were cloned into the plasmid pACYC Duet to express a recombinant and untagged version of these proteins. The cyclin genes from *L. major* used were CYCA, CYC3, CYC6, CYC7, CYC9 CYC10 and CYCA from *L. mexicana*.

4.2 Results

4.2.1 Sequence Alignments

All alignments and a phylogenetic trees were generated using Invitrogen program package Vector Nti wich includes Align X. Align X uses Clustal W algorithms.

4.2.1.1 Cyclins

The sequences of the major human cyclins and the sequences of trypanosomatid cyclins were analysed. A phylogenetic tree containing all *L. major* cyclins and the human cyclins was generated (Figure 4.1). These alignments show that LmCYCA and CYC6 are clustered together with human mitotic cyclins A and B. It shows that that CYC3 is most closely related to human cyclin E and that CYC8 and CYC9 are more related to human cyclins C and H, while other *L. major* cyclins like CYC2, 4, 5, 7, 10 and 11 do not appear have closer relation with any of these human cyclins analysed.

A phylogenetic tree of different Human Cyclins (A1, A2, B1, B2, C, D1, E1, E2 and H) and CYC9 from *L. brasiliensis, L. infantum, L. major* and *T. brucei* was built. The tree shows all *Leishmania* CYC9 proteins clustering close to transcriptional cyclins C and H. TbCYC9 which share only 30.1% identity with the *Leishmania* homologue also clusters with these transcriptional cyclins (Figure 4.2). An alignment of the protein sequence from TbCYC9, LmCYC9 and human transcriptional cyclins H and C is shown in Figure 4.3. The sequence alignment between human cyclin C, H and *L. major* CYC9 provided a low identity (17.6% for cyclin C and 13.4% for cyclin H). A sequence alignment between the three *Leishmania* CYC9 proteins and the *T. brucei* CYC9 (Figure 4.4) shows that this gene product is much more conserved between the *Leishmania* species (identity of 88.2% between *Leishmania* species) suggesting that this may be due to the existence of high

selective pressure on these genes and that this protein might have different function between *Leishmania* and *Trypanosoma* species.

4.2.1.2 CRKs

A sequence analysis of LmCRKs was carried out. A phylogenetic tree with LmCRKs and human CDK1 and CDK2 is shown in Figure 4.5. The tree shows that LmCRK3 is the protein from the CRK family that clusters with human CDK1 and CDK2. The similarity of all LmCRKs to human Cdc2 was analysed in the Table 4.1. In this table LmCRK3 has the highest level of sequence identity to both human Cdc2 and Yeast Cdc2 41.8% and 50.2% respectively. A sequence alignment using all LmCRKs is shown in Figure 4.6. In this alignment it can be observed that CRK1, CRK4 and CRK7 have a shorter N-terminus when compared to the other CRKs. CRK4 possesses two large insertions in central regions and an extended C-terminus. LmCRK8 has a large N-terminal and C-terminal insertion not found in any other CRK, it also possesses a small insertion in its central region. Finally, it is important to notice in the LmCRKs alignment the cyclin binding motif (PSTAIRE in Cdc2) is highly polymorphic (103aa-109aa) for an unknown reason.

Another important motif in the LmCRKs was analysed in Figure 4.7. The T-loop activation motif from all *Leishmania* CRKs was aligned. The threonine residue at this motif is phosphorylated by a kinase activating kinase (CAK/Civ1 in yeast and CDK7 in mammalian cells) is conserved in LmCRK1-3, LmCRK6 and LmCRK8. It is substituted by a serine residue in LmCRK4 and LmCRK7.

4.2.2 RT-PCR to detect the expression of cyclin genes in L. major

An RT-PCR to detect the expression of cyclin genes in *L. major* promastigotes was performed (Figure 4.8). Using cDNA obtained from total RNA extractions from promastitgote *L. major,* PCR reactions were performed to confirm the expression of the

cyclin genes in these cells. All PCRs used as sense primer the SL primer (OL1760) and a specific anti-sense primer specific for the cyclin sequence tested. It was possible to confirm the expression of LmCYCA, CYC7 and CYC9, while it was not possible to amplify the specific fragment from LmCYC3. For LmCYC10 a fragment that differed in size from that expected was obtained and therefore the expression could not be confirmed.

4.2.3 Cloning of L. major CYCs

To assay which cyclins might activate the different CRK in *L. major,* plasmids expressing tagged cyclins were made. The cloning of these proteins and expression attempts were described in previous chapters. The genes corresponding to *L. major* CYCA, CYC3, CYC6, CYC7, CYC9, CYC10 and CYC11 were amplified by PCR as described and cloned into pGEM-T vector. The genes were excised with *Nde*I and *Bam*HI restriction sites, gel purified and ligated into previously digested and gel purified pET15b vector generating pGL1334 (LmCYCA), pGL1218 (LmCYC6), pGL1335 (LmCYC7), pGL1336 (LmCYC9), pGL1337 (LmCYC10), pGL1350 (LmCYC11), pGL630 (LmeCYCA).

4.2.4 Purification of L. major cyclins

4.2.4.1 LmCYC3

The plasmid pGL1350, derived from pET-15b+ and containing the gene for LmCYC3, was used to express and affinity purify the LmCYC3his fusion protein. The plasmid was used to transform *E. coli* BL21 DE3 pLys, induced with IPTG overnight and standard histidine tagged protein purification was carried out. The result of this purification can be observed in Figure 4.9 (B). Lane 1 shows insoluble His tagged CYC3 around 46.9 kDa, which was also detected in a western blot using α-his antibody. No soluble protein could be purified and used in kinase assays from these preparations.

4.2.4.2 LmCYC7

The plasmid pGL1335, derived from pET-15b+ and containing the gene for LmCYC7, was used to express and affinity purify the LmCYC7his fusion protein. The plasmid was used to transform *E. coli* BL21 DE3 pLys, induced with IPTG overnight and standard histidine tagged protein purification was carried out. The result of this purification can be observed in Figure 4.9 (C). Lane 1 shows insoluble His tagged CYC7 around 27.6 kDa, which was also detected in a western blot using α-his antibody. No soluble protein could be purified and used in kinase assays from these preparations.

4.2.4.3 LmCYC10

The plasmid pGL1337, derived from pET-15b+ and containing the gene for LmCYC10 was used to express and affinity purify the LmCYC10his fusion protein. The plasmid was used to transform *E. coli* BL21 DE3 pLys, induced with IPTG overnight and standard histidine tagged protein purification was carried out. The result of this purification can be observed in Figure 4.9 (A). Lane 1 shows insoluble His tagged CYC10 around 68.7 kDa, which was also detected in a western blot using α-his antibody. No soluble protein could be purified and used in kinase assays from these preparations.

4.2.4.4 LmCYC9

The plasmid pGL1336, derived from pET-15b+ and containing the gene for was used to express and affinity purify the LmCYC9his fusion protein. The plasmid was used to transform *E. coli* BL21 DE3 pLys, induced with IPTG overnight and standard histidine tagged protein purification was carried out. The result of this purification can be observed in Figure 4.10. Lane 4 shows soluble His tagged CYC9 (32.9 kDa) purified at the concentration of 1.5mg/ml. Around 15mg of this protein could be obtained from 1L of cell culture

4.2.5 Purification of LmeCRK3:LmCYC6his complex

L. mexicana CRK3 was co-expressed in *E. coli* with CYC6his (Rod Walker, unpublished). The complex was purified from *E. coli* cell pellets by Nickel-chelate affinity chromatography. After purification with POROS MC 20 Column (Ni^{2+} charged), the samples were further purified by Ion-Exchange Chromatography and the elutions were loaded onto a Coomassie gel (Figure 4.11). A clear doublet corresponding to CYC6his and CRK3 can be observed in the elutions. A Histone H1 kinase assay demonstrated that the purified CRK3/CYC6his complex was active *in vitro*. This complex was used in further kinase assays, as a substrate and as a positive control.

4.2.6 Purification of CRK1-4 and CRK6-8

LmCRK genes were amplified from gDNA and subcloned in the expression vector pET-15b+, which was pre-digested with *Bam*HI and *Nde*I generating pGLs containing His tagged LmCRKs. The tagged proteins were successfully purified using pGL1342 for LmCRK8 (44.4 kDa), pGL1349 for CRK7 (32.4 kDa), pGL1341 for CRK6 (37.3 kDa), pGL1616 for CRK4 (51.7 kDa), pGL1340 for CRK3 (35 kDa), pGL1339 for CRK2 (36.4 kDa) and pGL1338 for CRK1 (34.4 kDa) (Figure 4.12 to 4.18). Soluble His tagged CRKs can be observed in lane 4 of Figures 4.12 to 4.18. These proteins were used in kinase assays with purified CYC9.

4.2.7 Cloning of untagged CRKs

To purify new recombinant *L. major* cyclin:kinase complexes it was necessary to design DNA constructs that could express these protein without a histidine tag. As described previously, it was possible to express untagged CRK3 and purify this protein from *E. coli* lysate using histidine tagged CYC6 or CYCA immobilized to an affinity column. The *L. major* CRKs were excised from their plasmids (see methods) using restriction sites *Bam*HI

and *Xho*I. DNAs fragments corresponding to the correct sizes were gel purified and ligated into dual expression vector pACYC Duet. This plasmid, which confers chloramphenicol resistance to the cells, was previously digested with *Bam*HI and *Xho*I and gel purified. pACYC Duet plasmids containing the *L. major* CRKs were named pGL1338 (CRK1), pGL1339 (CRK2), pGL1340 (CRK3), pGL1616 (CRK4), pGL1341 (CRK6), pGL1349 (CRK7), pGL1342 (CRK8) and pGL1072 (TbCRK3).

4.2.8 Co-transformation

Successful cloning of cyclins genes in pET15b and of CRKs into pACYC Duet allowed further co-transformation experiments to take place. pET15b derived pGLs containing cyclin genes (CYCA, CYC6, CYC7, CYC9, CYC10, CYC11) were transformed into BL21 *E. coli* cells and these were made ampicillin resistant competent cells. The cells were co-transformed with a second plasmid containing the CRK gene generating a double resistant *E. coli* strain capable of expressing one His-tagged CYC protein and one non-His-tagged CRK. The ampicillin resistance pET15b derived pGLs containing cyclins genes and the chloramphenicol pACYC Duet derived pGLs containing the CRKs generated double resistant cell lines. Each of the CYC containing cell line was transformed with a different CRK and in total, 24 different strains were generated. Each of the 24 cell lines contained two different plasmids, each capable of expressing a single cyclin and a single CRK. These cell lines were used in expression and purification assays.

4.2.9 Phosphorylation of the LmCRK T-loop threonine using Civ1

Each of the purified CRKs was tested as to whether they were substrates for Civ1. This was to assess if the threonine or serine residue in the activation loop would be phosphorylated by Civ1 in a similar manner as CRK3. CRKs 1-3, 6 and 8 have a threonine residue conserved in the activation loop, whereas CRKs 4 and 7 have a serine (Figure 4.7).

The only *L. major* CRK phosphorylated by Civ1 was CRK3 (Figures 4.19-4.21). Although CRK1, CRK2, CRK6 and CRK8 have a conserved threonine residue, like CRK3, they could not be phosphorylated by Civ1. Although there is no evidence that Civ1 targets serines, the serine residue in CRK7 and CRK4 have the potential to be phosphorylated by Civ1 since this protein is a serine/threonine kinase. Nevertheless these proteins were not phosphorylated by Civ1 (Figures 4.19-4.21).

4.2.10 Co-expressions and purifications

Co-transformed *E. coli* BL21 cells capable of expressing one his-tagged cyclin protein and one non-his-tagged CRK were grown to 0.6 OD. in the presence of ampicilin and chloramphenicol antibiotics. When an O.D. of 0.6 was reached, the culture was transferred to 19^{o}C and induced for protein expression using 1mM IPTG overnight shaking for better aeration. From the total culture volume of 200ml, half was used to co-purify cyclin-kinase complexes using the same protocol used for purification of CRK3.

All cell lines containing different kinases and LmCYCA, LmeCYCA, LmCYC6, LmCYC7 and LmCYC9 were tested and the co-purification attempted. All of the co-purifications were inconclusive and none of the 35 attempts generated a kinase cyclin pair as had been observed previously for CYC6:CRK3. Most of the purifications appeared to follow the same pattern, where no eluted protein could be detected (Figure 4.22). Another similar pattern can be observed in Figure 4.23, where the cyclin appears to be insoluble and is detected in lane 1. However no cyclin or CRK was eluted in lanes 5 and 6, as observed in Figure 4.22. Finally, the last pattern observed is shown in Figure 4.24, where a band corresponding to the size of the CRKs is detected in the elution, but no band corresponding to the cyclin was observed as it would be expected. This was observed only in one of the purifications, containing LmCYC7 and LmCRK1, suggesting that the kinase was eluted. However, the apparent absence of the cyclin in the eluted fraction requires explanation.

4.2.11 Kinase assays using CYC9 to activate LmCRKs

All CRKs were tested for autophosphorylation and for cyclin-independent activity against three different substrates, histone H1, MBP and β-Casein. None of the kinases tested had autophosphorylation activity or activity towards the substrates tested on their own. Protein kinase assays using CYC9 to activate different *L. major* CRKs were performed as described previously. CYC9 was used to activate CRK1, CRK2, CRK3, CRK4, CRK6, CRK7 and CRK8. HistoneH1, MBP and β-Casein were used as substrates. No activity from these kinases was observed against any of the substrates used. When Civ1 was added to the kinase assays, the presence of CYC9 did not activate the complex and Civ1 did not phosphorylate any of the aforementioned CRKs as previously reported.

4.2.12 Kinase assays of co-eluted purifications

Elutions from co-expressed purifications were assayed *in vitro* kinase assays. Figure 4.25 shows the result of a kinase assay performed with two different kinases and CYC7 (CRK1 + CYC7 and CRK6 + CYC7) against three different substrates Histone H1, β-Casein and MBP. Reactions were performed in kinase assay buffer containing 5µg of histone H1 or β-Casein or MBP as substrate and γ-ATP labelled with P^{32}. The samples were analysed on 12% SDS-PAGE gel, stained with Coomassie, dried and exposed to a ^{32}P particle emission sensitive film. As a positive control, the active CRK3:CYC6his complex was used. It was observed that these purifications contained no active cyclin:kinase complexes against the substrates used (Figure 4.25), while the complex LmeCRK3:LmCYC6his was shown to phosphorylate Histone H1, (Figure 3.13) as well as MBP and β-casein (Figure 4.25).

4.2.13 Western blot and kinase assay of in vivo expressed HA epitope tagged LmCRK6

L. major cell lines were transfected with pGL1392 and pGL1394. For pGL1392 a C-terminal HA tag was added by PCR to LmCRK6 for overexpression in an episomal plasmid. For pGL1394 a C-terminal HA tagged LmCRK6 was expressed by integration of the construct into the rDNA locus. The integration plasmid was generated from pRIB plasmid backbone that was digested with *PacI* and *PmeI* before transfection of *L. major*. Lysates from Wild type cells and from the transfected cell lines were prepared and S-100 soluble extracts were used in Western blots using α-HA antibody (Figure 4.26). The HA tagged CRK6 was detected in the western blot for both overexpressor and integrated cell lines, while no protein was detected in wild type cells. The result from the western blot suggested that LmCRK6 was successfully tagged and could be purified. The same S-100 was used for immunoprecipitation of HA tagged proteins using a HA affinity matrix (Roche). After washes the matrix containing the active kinase was tested for kinase activity (Figure 4.26). Kinase activity was detected using Histone H1 as substrate for both overexpressor and integrated cell lines, while no activity was detected after affinity purification from wild type cells.

4.2.14 Immunofluorescense assay for cell lines expressing LmCRK6 HA tagged

An Immunofluorescense assay to detect LmCRK6HA was performed in the transfected *L. major* cell lines and in the wild type cells. The primary antibody, α-HA Roche mouse monoclonal was diluted 1 in 500 in 0.1% Triton X-100, 0.1% BSA and added to the top of the slide to incubate for 1 hour at room temperature The secondary antibody was α-mouse FITC conjugated diluted at 1 in 1,000. A cytoplasmic localisation was observed in both WCMP 7653 (*L. major* containing pGL1392) and WCMP 7654 (pGL1394 integrated into

L. major), which was not observed in the wild type cells (Figure 4.27). This suggests that LmCRK6 is present in the cytoplasm of *L. major* cells throughout the cell.

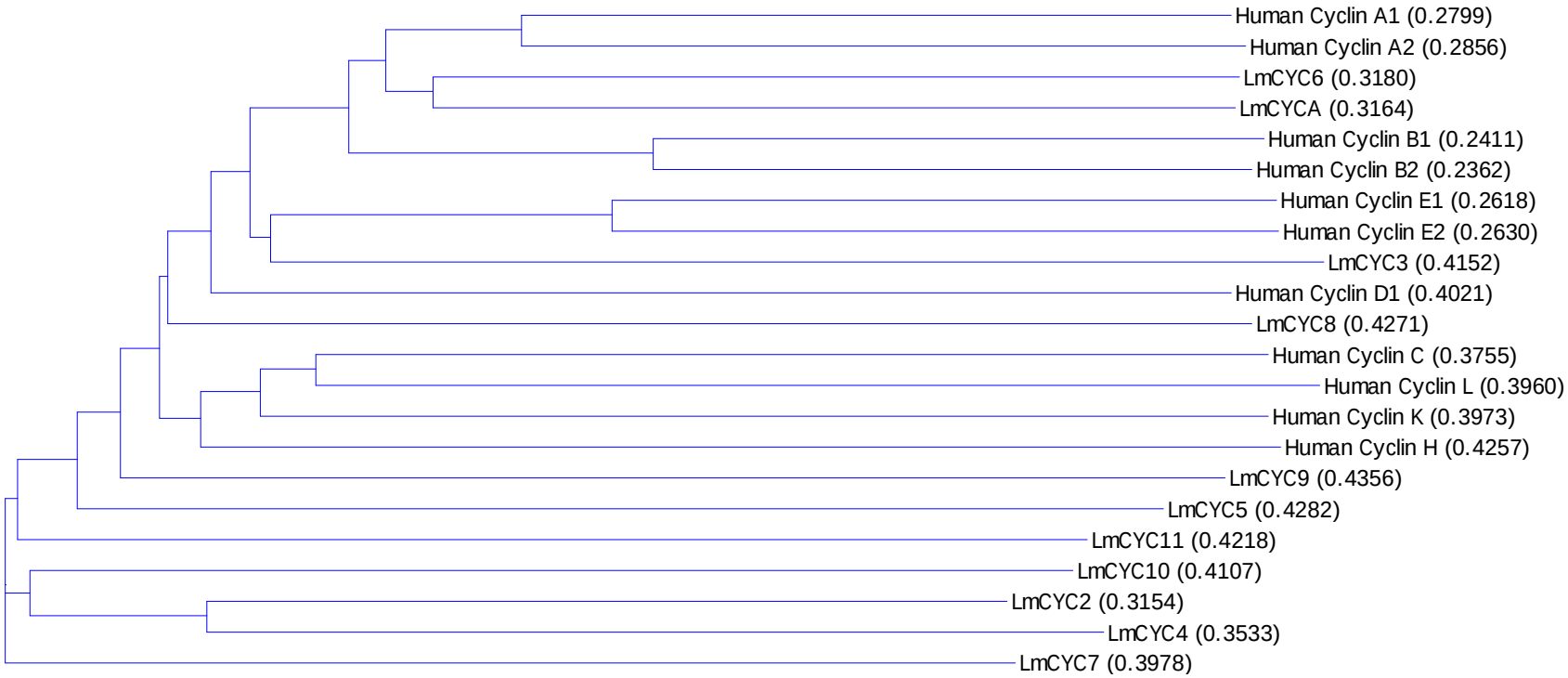


Figure 4.1 Phylogenetic tree of different Human Cyclins cyclins and *L. major* CYCs. From Human: A1, A2, B1, B2, C, D1, E1, E2, H and L. From *L. major* CYC2-11 and CYCA. Sequences from gene bank accession numbers are NM_057749 for human cyclin E2, NM_001238 for human cyclin E1, NM_004701 for human cyclin B2, NM_031966 for human cyclin B1, NM_001237 for human cyclin A2, NM_003914 for human cyclin A1, NM_053056 for human cyclin D1, NM_005190 for human cyclin C, NM_001239 for human cyclin H, LmjF32.0760 and NM_020307 for human cyclin L. for *L. major* gene bank accession numbers: in the order CYC2-11 and CYCA; LmjF32.0820, LmjF30.0080, LmjF05.0710, LmjF33.0770, LmjF32.3320, LmjF30.3630, LmjF26.0330, LmjF32.0760, LmjF24.1890, LmjF24.1880 and LmjF25.1470. Lines represent the degree of divergence between the sequences while the calculated distance values are shown in parenthesis.

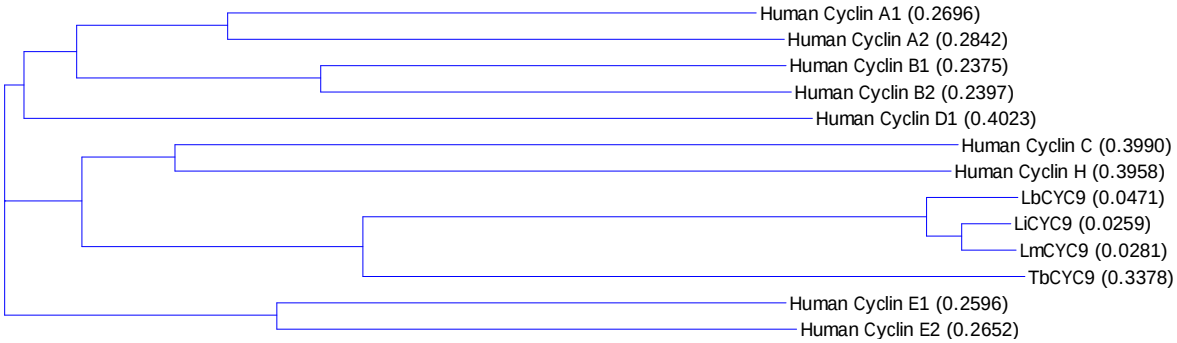


Figure 4.2 Phylogenetic tree of different cyclins. Human Cyclins A1, A2, B1, B2, C, D1, E1, E2 and H were used. CYC9 from *L. brasiliensis*, *L. infantum*, *L. major* and *T. brucei* were also used. Sequences from gene bank accession numbers are NM_057749 for human cyclin E2, NM_001238 for human cyclin E1, NM_004701 for human cyclin B2, NM_031966 for human cyclin B1, NM_001237 for human cyclin A2, NM_003914 for human cyclin A1, NM_053056 for human cyclin D1, NM_005190 for human cyclin C, NM_001239 for human cyclin H, LmjF32.0760 for *L. major* CYC9, Tb11.01.5600 for *T. brucei* CYC9, LinJ32_V3.0800 for *L. infantum* CYC9 and LbrM32_V2.0850 for *L. braziliensis* CYC9. Lines represent the degree of divergence between the sequences while the calculated distance values are shown in parenthesis.

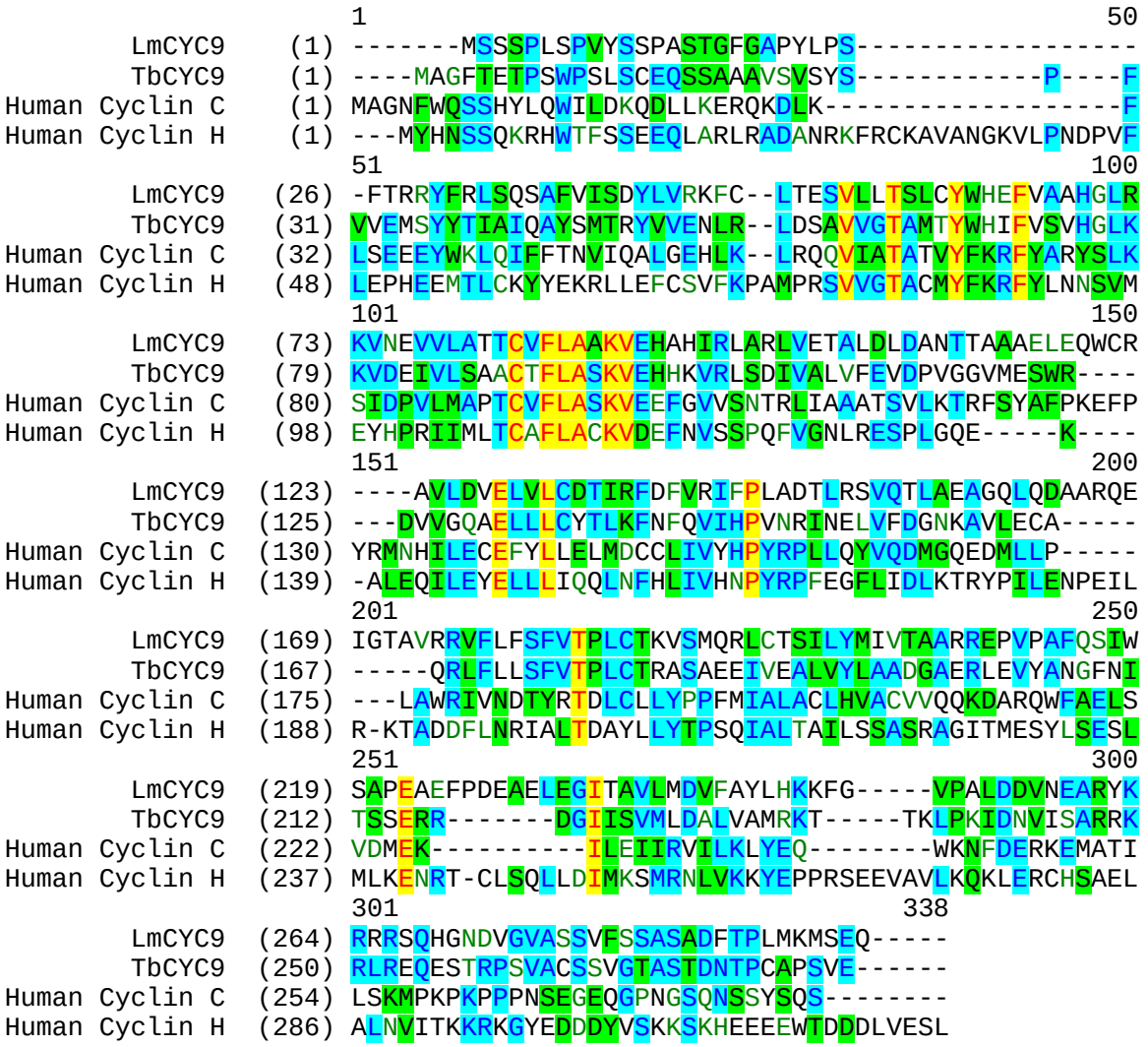


Figure 4.3 Sequence alignment of human cyclin C (NM_005190), *L. major* CYC9 (LmjF32.0760) and *T.brucei* CYC9 (Tb11.01.5600). The sequence identity between LmCYC9 and TbCYC9 is low (30.1%). When LmCYC9 is compared to Human cyclin C and cyclin H the sequence identity between then is 17.9% and 11.9% respectively. In the alignment the yellow colour represents the amino acids residues that are conserved throughout all species, the blue colour represents amino acids residues that are conserved throughout most of the species analysed but not in all of them. Finally, the amino acid residues that are poorly conserved throughout all the species are represented in green.

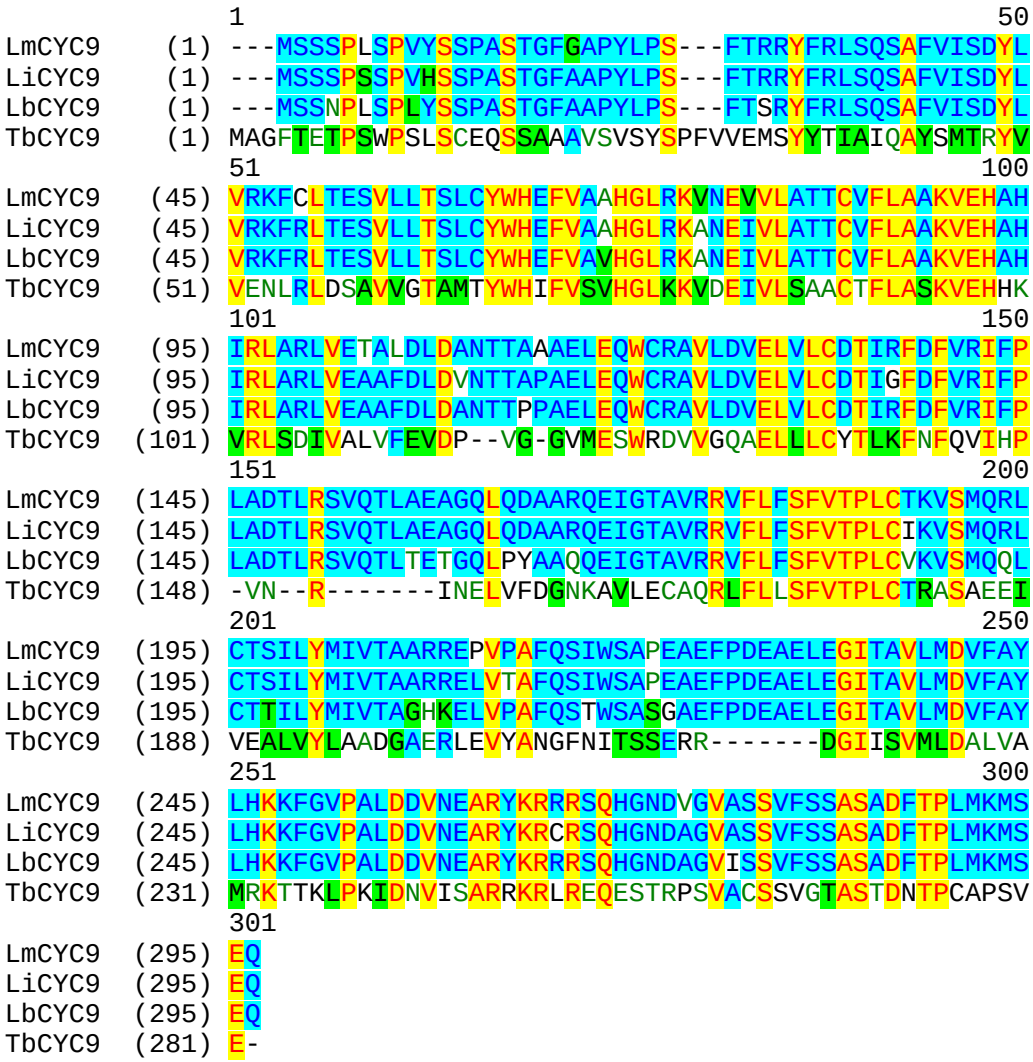


Figure 4.4 Sequence alignment of different CYC9 proteins from trypanosomatid. From line 1 to line 4, *L. major* CYC9 (LmjF32.0760), *L. infantum* CYC9 (LinJ32_V3.0800), *L. brasiliensis* CYC9 (LbrM32_V2.0850), and *T. brucei* CYC9 (Tb11.01.5600). In the alignment the yellow colour represents the amino acids residues that are conserved throughout all species, the blue colour represents amino acids residues that are conserved throughout most of the species analysed but not in all of them. Finally, the amino acid residues that are poorly conserved throughout all the species are represented in green.

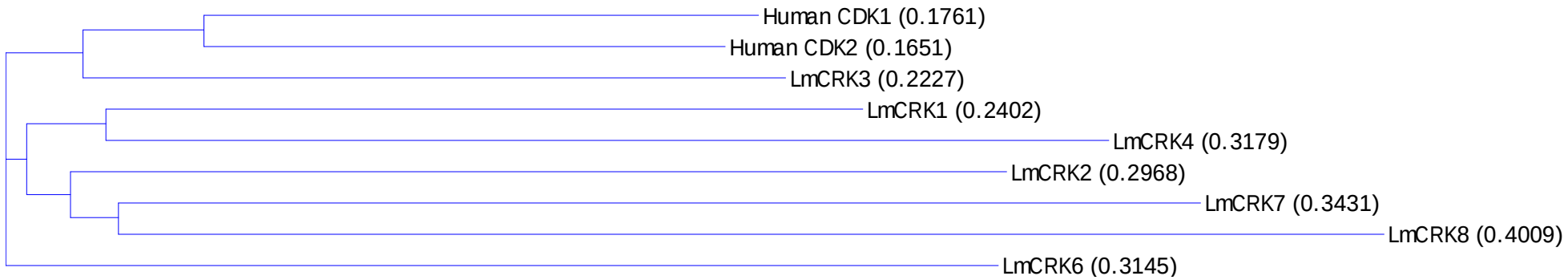


Figure 4.5 Phylogenetic tree of *Leishmania* major CRKs and human CDKs. Human CDK1 and CDK2, as well as *L. major* CRK1, 2, 3, 4, 6, 7 and 8 were used. Sequences from gene bank accession numbers are NP_001777 for human CDK1, NM_001798 for human CDK2, LmjF21.1080 for LmCRK1, LmjF05.0550 for LmCRK2, LmjF36.0550 for LmCRK3, LmjF16.0990 for LmCRK4, LmjF27.0560 for LmCRK6, LmjF26.0040 for LmCRK7 and LmjF11.0110 for LmCRK8. Lines represent the degree of divergence between the sequences while the calculated distance values are shown in parenthesis.

Table 4.1 Sequence identity comparison between *L. major* CRKs compared to yeast Cdc2. Accession numbers for *L. major* proteins are CRK1 (LmjF21.1080), LmCRK2 (LmjF05.0550), LmCRK3 (LmjF36.0550), LmCRK4 (LmjF16.0990), LmCRK6 (LmjF27.0560), LmCRK7 (LmjF26.0040), LmCRK8 (LmjF11.0110), Yeast (*S.pombe*) cell division gene (CDC2) (M12912) and human Cdc2 (NM_004196).

	LmCRK1	LmCRK2	LmCRK3	LmCRK4	LmCRK6	LmCRK7	LmCRK8
S. pombe Cdc2	47.6%	39.4%	50.2%	24.4%	39.9%	34.4%	18.8%
H. sapiens Cdc2	31.1%	26.4%	41.8%	18.9%	25.5%	25.1%	17.7%

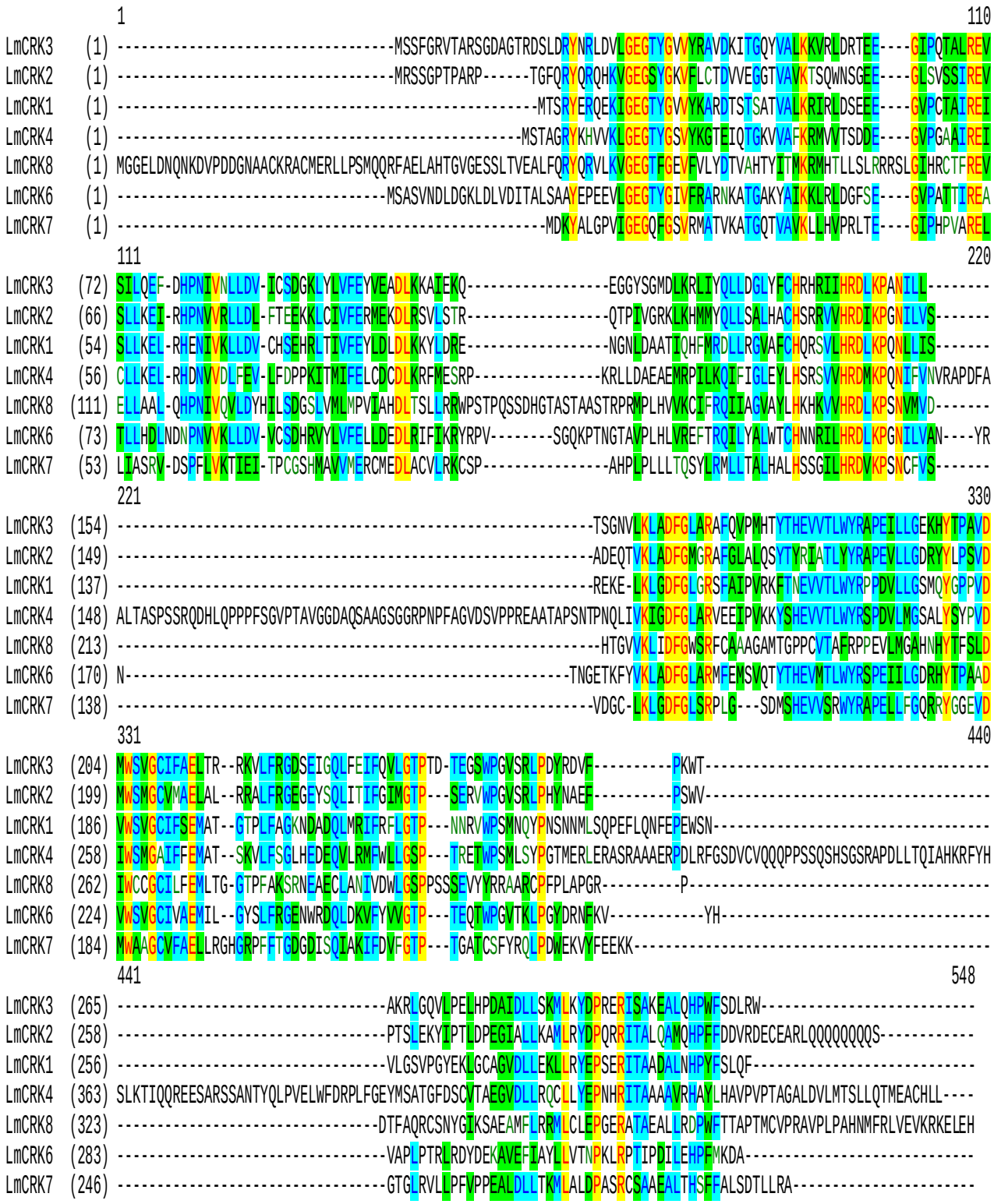

1 110
LmCRK3 (1) MSSFGRVTARSGDAGTRDSLDRYNRLDVLGEGTYGVVYRAVDKITGQYVALKKVRLDRTEE GIPQTALREV
LmCRK2 (1) MRSSGPTPARP TGFQRYQRQHKVGEGSYGKVFLCTDVVEGGTVAVKTSQWNSGEE GLSVSSIREV
LmCRK1 (1) MTSRYERQEKIGEGTYGVVYKARDTSTSATVALKRIRLDSEEE GVPCTAIREI
LmCRK4 (1) MSTAGRYKHVVKLGEGTYGSVYKGTEIQTGKVVAFKRMVVTSDDE GVPGAAIREI
LmCRK8 (1) MGGELDNQNKDVPDDGNAACKRACMERLLPSMQQRFAELAHTGVGESSLTVEALFQRYQRVLKVGEGTFGEVFVLYDTVAHTYITMKRMHTLLSLRRRSLGIHRCTFREV
LmCRK6 (1) MSASVNDLDGKLDLVDITALSAAYEPEEVLGEGTYGIVFRARNKATGAKYAIKKLRLDGFSE GVPATTIREA
LmCRK7 (1) MDKYALGPVIGEGQFGSVRMATVKATGQTVAVKLLHVPRLTE GIPHPVAREL
111 220
LmCRK3 (72) SILQEF-DHPNIVNLLDV-ICSDGKLYLVFEYVEADLKKAIEKQ EGGYSGMDLKRLIYQLLDGLYFCHRHRIIHRDLKPANILL
LmCRK2 (66) SLLKEI-RHPNVVRLLDL-FTEEKKLCIVFERMEKDLRSVLSTR QTPIVGRKLKHMMYQLLSALHACHSRRVVHRDIKPGNILVS
LmCRK1 (54) SLLKEL-RHENIVKLLDV-CHSEHRLTIVFEYLDLDLKKYLDRE NGNLDAATIQHFMRDLLRGVAFCHQRSVLHRDLKPQNLLIS
LmCRK4 (56) CLLKEL-RHDNVVDLFEV-LFDPPKITMIFELCDCDLKRFMESRP KRLLDAEAEMRPILKQIFIGLEYLHSRSVVHRDMKPQNIFVNVRAPDFA
LmCRK8 (111) ELLAAL-QHPNIVQVLDYHILSDGSLVMLMPVIAHDLTSLLRRWPSTPQSSDHGTASTAASTRPRMPLHVVKCIFRQIIAGVAYLHKHKVVHRDLKPSNVMVD
LmCRK6 (73) TLLHDLNDNPNVVKLLDV-VCSDHRVYLVFELLDEDLRIFIKRYRPV SGQKPTNGTAVPLHLVREFTRQILYALWTCHNNRILHRDLKPGNILVAN YR
LmCRK7 (53) LIASRV-DSPFLVKTIEI-TPCGSHMAVVMERCMEDLACVLRKCSP AHPLPLLLTQSYLRMLLTALHALHSSGILHRDVKPSNCFVS
221 330
LmCRK3 (154) TSGNVLKLADFGLARAFQVPMHTYTHEVVTLWYRAPEILLGEKHYTPAVD
LmCRK2 (149) ADEQTVKLADFGMGRAFGLALQSYTYRIATLYYRAPEVLLGDRYYLPSVD
LmCRK1 (137) REKE-LKLGDFGLGRSFAIPVRKFTNEVVTLWYRPPDVLLGSMQYGPPVD
LmCRK4 (148) ALTASPSSRQDHLQPPPFSGVPTAVGGDAQSAAGSGGRPNPFAGVDSVPPREAATAPSNTPNQLIVKIGDFGLARVEEIPVKKYSHEVVTLWYRSPDVLMGSALYSYPVD
LmCRK8 (213) HTGVVKLIDFGWSRFCAAAGAMTGPPCVTAFRPPEVLMGAHNHYTFSLD
LmCRK6 (170) N TNGETKFYVKLADFGLARMFEMSVQTYTHEVMTLWYRSPEIILGDRHYTPAAD
LmCRK7 (138) VDGC-LKLGDFGLSRPLG SDMSHEVVSRWYRAPELLFGQRRYGGEVD
331 440
LmCRK3 (204) MWSVGCIFAELLTR--RKVLFRGDSEIGQLFEIFQVLGTPTD-TEGSWPGVSRLPDYRDVF PKWT
LmCRK2 (199) MWSMGCVMAELLAL--RRALFRGEGEYSQLITIFGIMGTP SERVWPGVSRLPHYNAEF PSWV
LmCRK1 (186) VWSVGCIFSEMAT--GTPLFAGKNDADQLMRIFRFLGTP NNRVWPSMNQYPNSNNMLSQPEFLQNFEPEWSN
LmCRK4 (258) IWSMGAIFFEMAT--SKVLFSGLHEDEQVLRMFWLLGSP TRETWPSMLSYPGTMERLERASRAAAERPDLRFGSDVCVQQQPPSSQSHSGSRAPDLLTQIAHKRFYH
LmCRK8 (262) IWCCGCILFEMLTG-GTPFAKSRNEAECLANIVDWLGSPPSSSEVYYRRAARCPFPLAPGR P
LmCRK6 (224) VWSVGCIVAEMIL--GYSLFRGENWRDQLDKVFYVVGTP TEQTWPGVTKLPGYDRNFKV YH
LmCRK7 (184) MWAAGCVFAELLRGHGRPFFTGDGDISQIAKIFDVFGTP TGATCSFYRQLPDWEKVYFEEKK
441 548
LmCRK3 (265) AKRLGQVLPELHPDAIDLLSKMLKYDPRERISAKEALQHPWFSDLRW
LmCRK2 (258) PTSLEKYIPTLDPEGIALLKAMLRYDPQRRITALQAMQHPFFDDVRDECEARLQQQQQQQQS
LmCRK1 (256) VLGSVPGYEKLGCAGVDLLEKLLRYEPSERITAADALNHPYFSLQF
LmCRK4 (363) SLKTIQQREESARSSANTYQLPVELWFDRPLFGEYMSATGFDSCVTAEGVDLLRQCLLYEPNHRITAAAAVRHAYLHAVPVPTAGALDVLMTSLLQTMEACHLL
LmCRK8 (323) DTFAQRCSNYGIKSAEAMFLRRMLCLEPGERATAEALLRDPWFTTAPTMCVPRAVPLPAHNMFRLVEVKRKELEH
LmCRK6 (283) VAPLPTRLRDYDEKAVEFIAYLLVTNPKLRPTIPDILEHPFMKDA
LmCRK7 (246) GTGLRVLLPFVPPEALDLLTKMLALDPASRCSAAEALTHSFFALSDTLLRA

Figure 4.6. Sequence alignment between *L. major* CRK1-4 and 6-7. Sequences from gene bank accession numbers are LmjF21.1080 for LmCRK1, LmjF05.0550 for LmCRK2, LmjF36.0550 for LmCRK3, LmjF16.0990 for LmCRK4, LmjF27.0560 for LmCRK6, LmjF26.0040 for LmCRK7 and LmjF11.0110 for LmCRK8. In the alignment the yellow colour represents the amino acids residues that are conserved throughout all species, the blue colour represents amino acids residues that are conserved throughout most of the species analysed but not in all of them. Finally, the amino acid residues that are poorly conserved throughout all the species are represented in green.

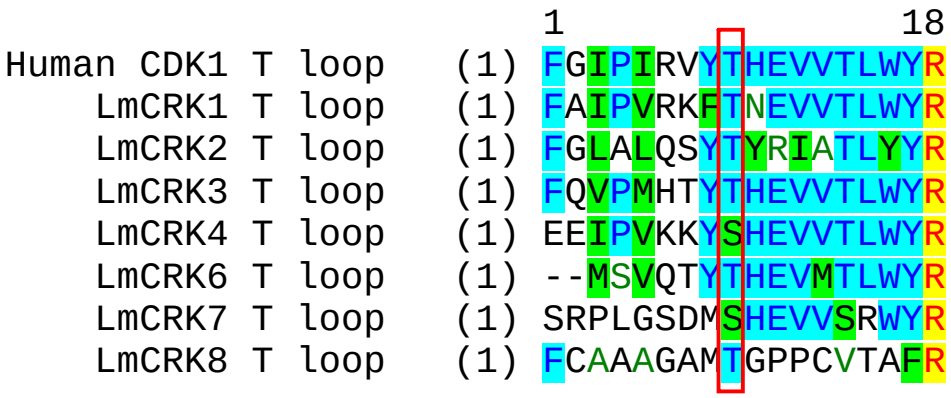


Figure 4.7 Sequence alignments between T-loop regions of human CDK1 and *L. major* CRK1-4 and 6-8. Sequences from gene bank accession numbers are NP_001777 for human CDK1, LmjF21.1080 for LmCRK1, LmjF05.0550 for LmCRK2, LmjF36.0550 for LmCRK3, LmjF16.0990 for LmCRK4, LmjF27.0560 for LmCRK6, LmjF26.0040 for LmCRK7 and LmjF11.0110 for LmCRK8. The alignment shows that the threonine residue, which is targeted for phosphorylation by CAK1, is conserved in LmCRK1, 2, 3, 6 and 8. It also shows that for CRK4 and CRK7 the threonine residue is substituted for a serine residue. In the alignment the yellow colour represents the amino acids residues that are conserved throughout all species, the blue colour represents amino acids residues that are conserved throughout most of the species analysed but not in all of them. Finally, the amino acid residues that are poorly conserved throughout all the species are represented in green.

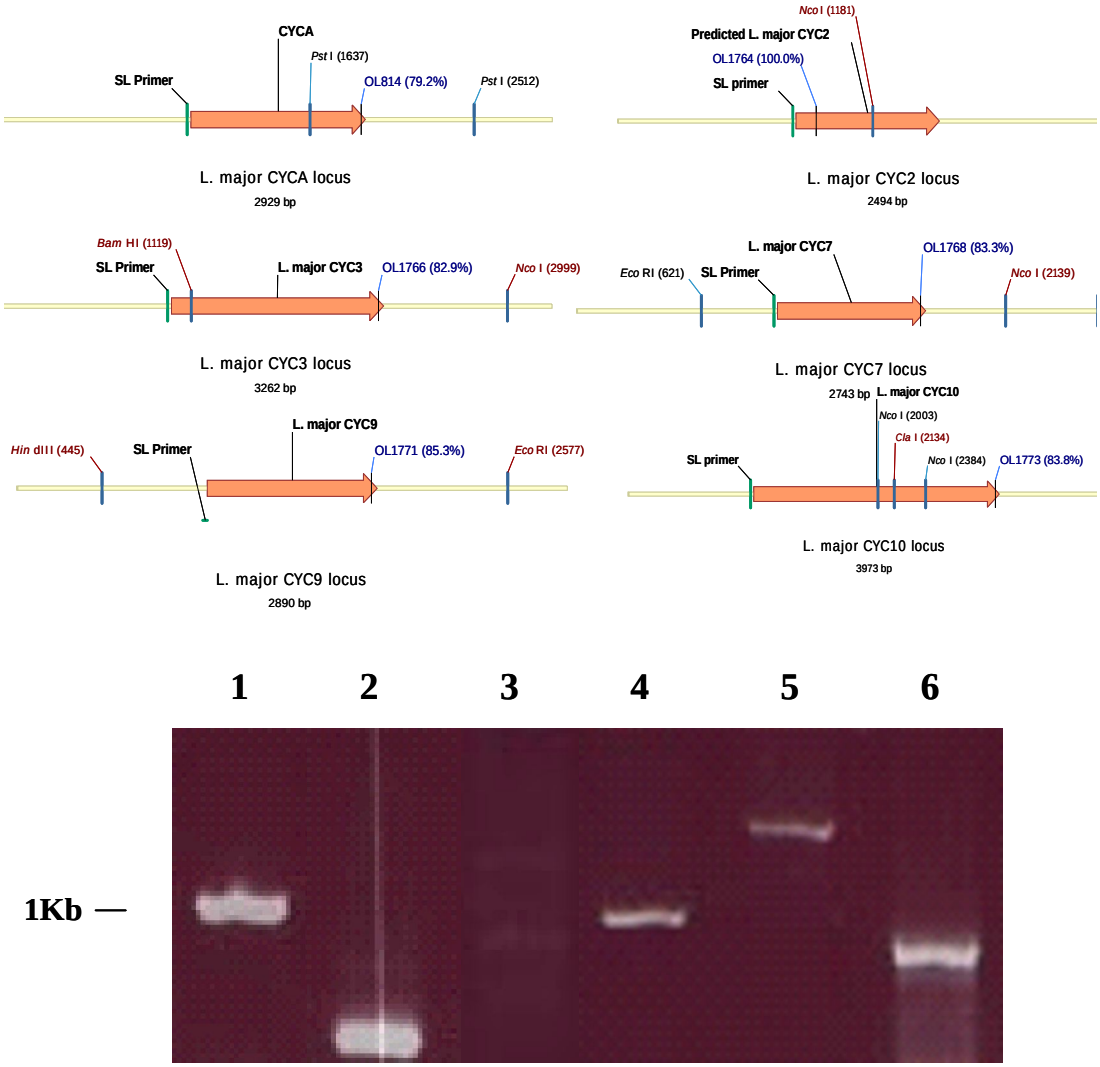


Figure 4.8. RT-PCR to detect the expression of cyclin genes in *L. major* promastigotes. Using cDNA from *L. major* PCR reactions were performed to confirm the expression of the cyclin genes in these cells. All PCRs used as sense primer the SL primer (OL1760) In lane 1, PCR using oligos OL1760/OL814 that amplify 1Kb from CYCA gene; In lane 2, PCR using oligos OL1760/OL1764 that amplify 0.6Kb fragment of *CYC2*; In lane 3, PCR using oligos OL1760/OL1766 that did not amplify a fragment of 1.2 Kb from *CYC3*; in lane 4, PCR using oligos OL1760/OL1768 that amplify a 0.8Kb fragment from the *CYC7*; in lane 5, PCR reaction using oligos OL1760/OL1771 that amplify a 1.1Kb fragment of *CYC9*; in lane 6, PCR reaction using oligos OL1764/OL1773 that did not amplify a fragment of 2Kb from the gene CYC10.

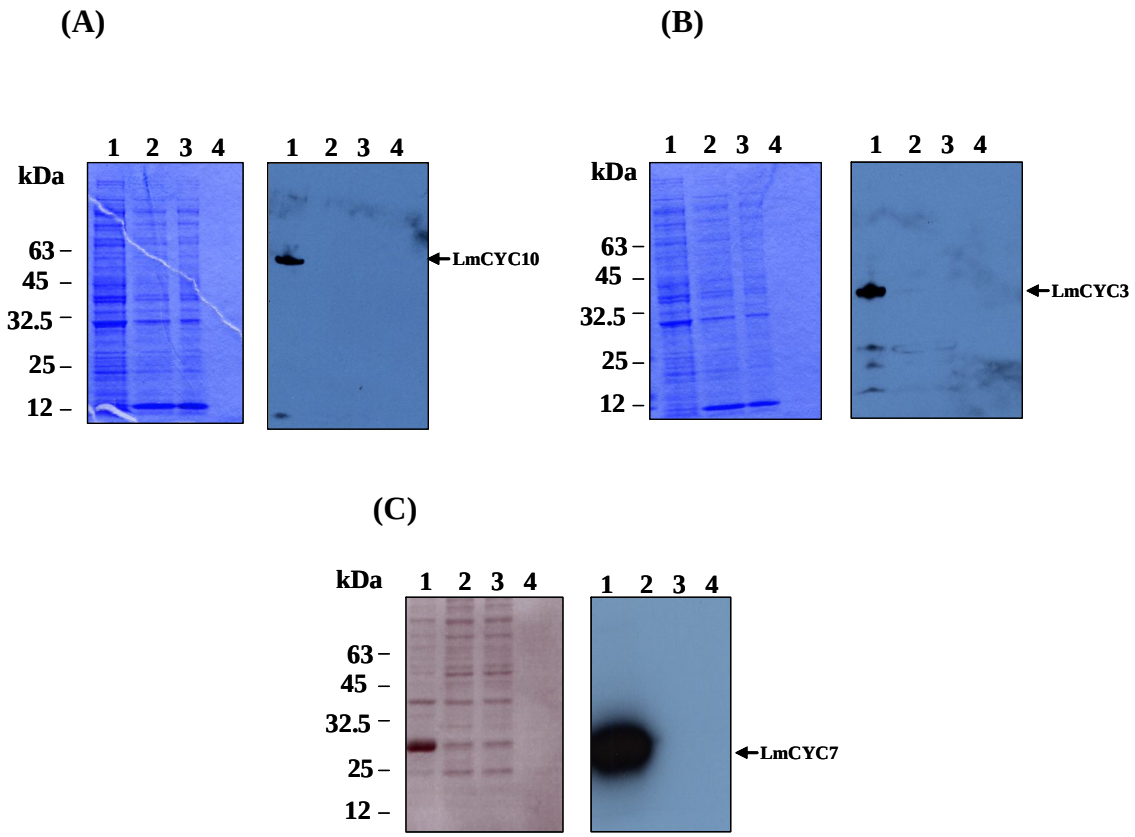


Figure 4.9 Attempt to purify *L. major* CYC proteins. LmCYC10, LmCYC3 and LmCYC7 were cloned into the expression vectors pGL1337, pGL1350, and pGL1335 respectively and were used for affinity purification with the Ni-Agarose column. The procedure used to purify LmCRK3his, was also used for the proteins above. The purification of the induced proteins was carried out and analyzed on SDS-PAGE gel stained with Coomassie Blue (Lanes 1-4). Lane 1: Insoluble fraction. Lane 2: Soluble fraction. Lane 3: Flow through. Lane 4: Elution. Western blot carried out with mouse α-his antibody diluted 1:5.000 as primary antibody and α-mouse HPR conjugated antibody as secondary, diluted 1:5.000. The proteins are arrowed and can be observed only in the insoluble Lane 1.

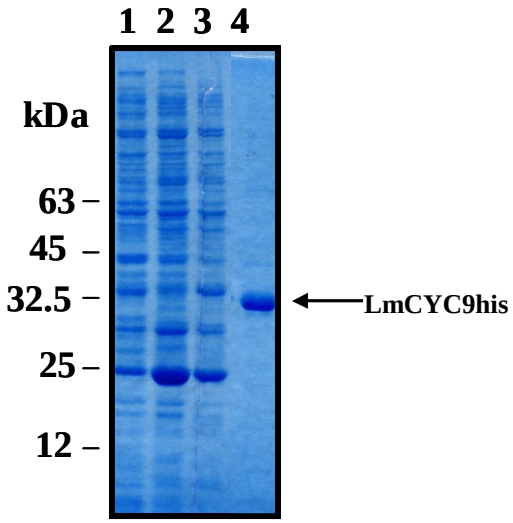


Figure 4.10 Purification of *L. major* CYC9. The same procedure used to purify LmCRK3his was used for LmCYC9his. The purification of the induced protein was carried out and analyzed on SDS-PAGE gel stained with Coomassie Blue (Lanes 1-4). Lane 1: Insoluble fraction. Lane 2: Soluble fraction. Lane 3: Flow through. Lane 4: Elution. A protein of around 34kDa (arrowed) can be observed in the Coomassie gel, which corresponds to LmCYC9his.

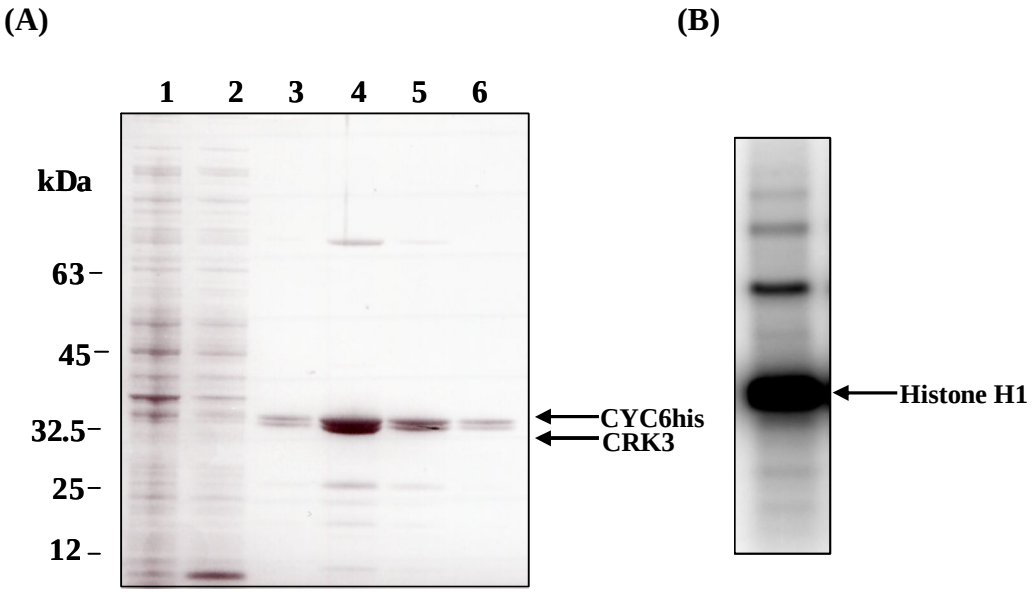


Figure 4.11 *L. mexicana* CRK3: *L. major* CYC6his purification and kinase assay. (A) *E. coli* BL-21 DE3 pLYS cells were used to induce the expression of LmCRK3 from pGL1072 and CYC6his from pGL1218. The purification of the induced protein was carried out and analyzed on an SDS-PAGE gel stained with Coomassie Blue (Lanes 1-6). Lane 1: Insoluble fraction. Lane 2: Soluble fraction. Lane 3 to 6: obtained from the flow through of the ion exchange purification. (B) A kinase assay shows Histone H1 band phosphorylated by the purified complex.

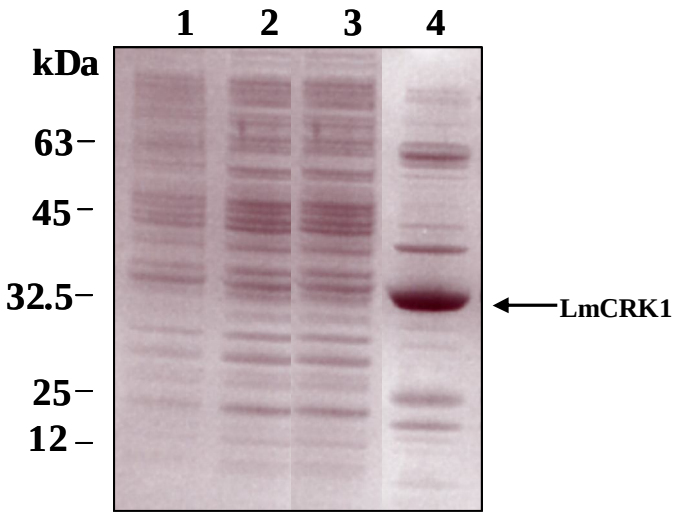


Figure 4.12 Purification of *L. major* CRK1. The same procedure used to purify LmCRK3his was used for LmCRK1his. The purification was analyzed on SDS-PAGE gel stained with Coomassie Blue (Lanes 1-4). Lane 1: Insoluble fraction. Lane 2: Soluble fraction. Lane 3: Flow through. Lane 4: Elution. A protein of 34.4kDa can be observed in the Coomassie (arrowed) which corresponds to LmCRK1his.

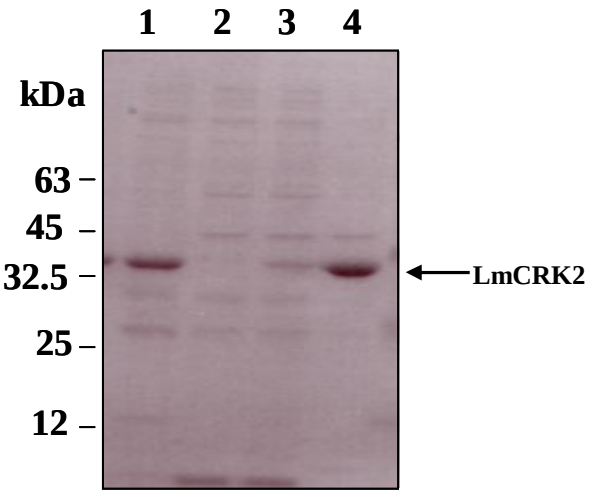


Figure 4.13 Purification of *L. major* CRK2. The same procedure used to purify LmCRK3his was used for LmCRK2his protein. The purification of the induced protein was carried out and analyzed on SDS-PAGE gel stained with Coomassie Blue (Lanes 1-4). Lane 1: Insoluble fraction. Lane 2: Soluble fraction. Lane 3: Flow through. Lane 4: Elution. A protein of 36.4kDa can be observed in the Coomassie (arrowed), which corresponds to LmCRK2his.

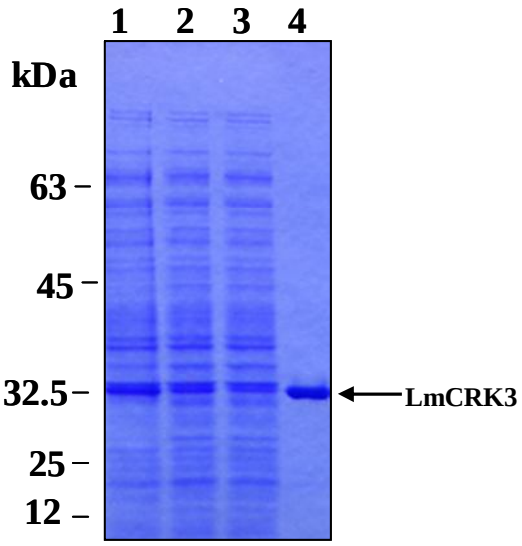


Figure 4.14 Purification of *L. major* CRK3. The same procedures used to purify LmeCRK3his were used for LmCRK3his. The purification of the induced protein was carried out and analyzed on SDS-PAGE gel stained with Coomassie Blue (Lanes 1-4). Lane 1: Insoluble fraction. Lane 2: Soluble fraction. Lane 3: Flow through. Lane 4: Elution. A protein of 35kDa can be observed in the Coomassie (arrowed), which corresponds to LmCRK3his.

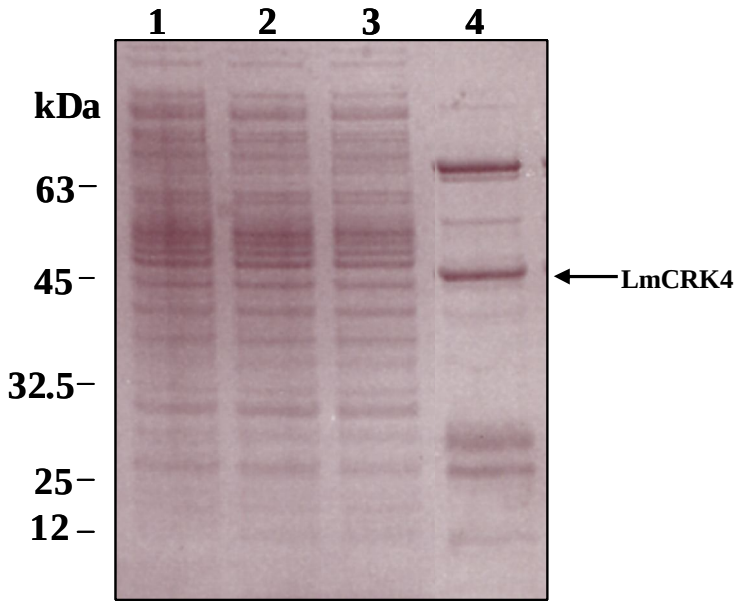


Figure 4.15 Purification of *L. major* CRK4. The same procedures used to purify LmCRK3his were used for LmCRK4his proteins of 51.7 kDa. The purification of the induced protein was carried out and analyzed on SDS-PAGE gel stained with Coomassie Blue (Lanes 1-4). Lane 1: Insoluble fraction. Lane 2: Soluble fraction. Lane 3: Flow through. Lane 4: Elution. A protein of 51.7kDa can be observed in the Coomassie (arrowed) which corresponds to LmCRK4his.

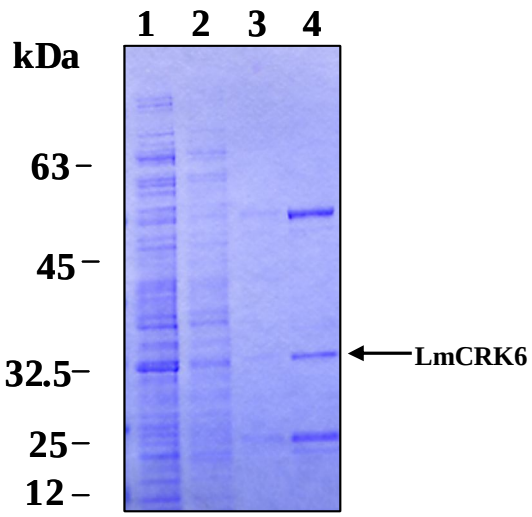


Figure 4.16 Purification of *L. major* CRK6. The same procedures used to purify LmCRK3his were also used for LmCRK6his protein of 37.3 kDa. The purification of the induced protein was carried out and analyzed on SDS-PAGE gel stained with Coomassie Blue (Lanes 1-4). Lane 1: Insoluble fraction. Lane 2: Soluble fraction. Lane 3: Flow through. Lane 4: Elution. A protein of 37.3kDa can be observed in the Coomassie (arrowed) which corresponds to LmCRK6his.

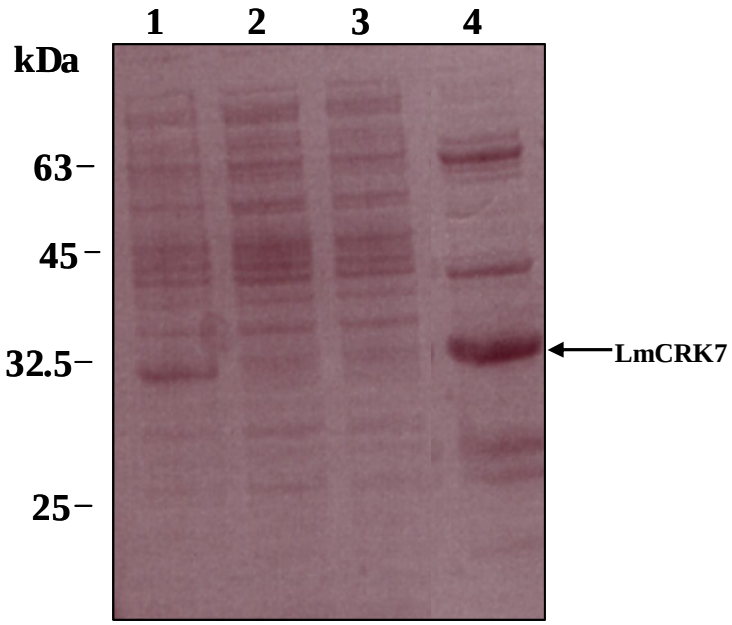


Figure 4.17 Purification of *L. major* CRK7. The same procedures used to purify LmCRK3his were also used for LmCRK7his protein of around 32.4 kDa. The purification of the induced protein was carried out and analyzed on SDS-PAGE gel stained with Coomassie Blue (Lanes 1-4). Lane 1: Insoluble fraction. Lane 2: Soluble fraction. Lane 3: Flow through. Lane 4: Elution. A protein of 32.4kDa can be observed in the Coomassie (arrowed) which corresponds to LmCRK6his.

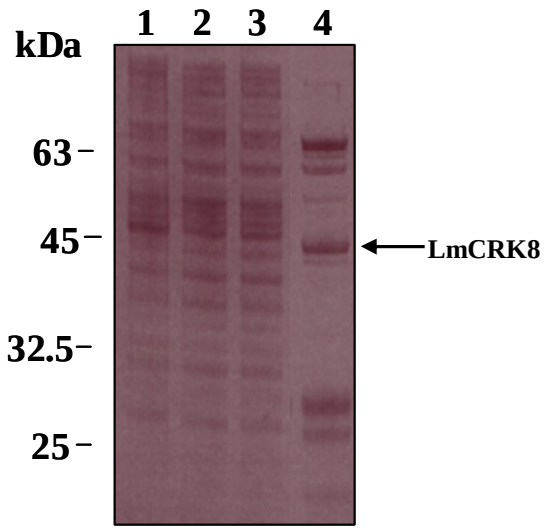


Figure 4.18 Purification of *L. major* CRK8. The same procedures used to purify LmCRK3his were also used for LmCRK8his protein of around 44.4 kDa. The purification of the induced protein was carried out and analyzed on SDS-PAGE gel stained with Coomassie Blue (Lanes 1-4). Lane 1: Insoluble fraction. Lane 2: Soluble fraction. Lane 3: Flow through. Lane 4: Elution. A protein of 44.4kDa can be observed in the Coomassie (arrowed) which corresponds to LmCRK6his.

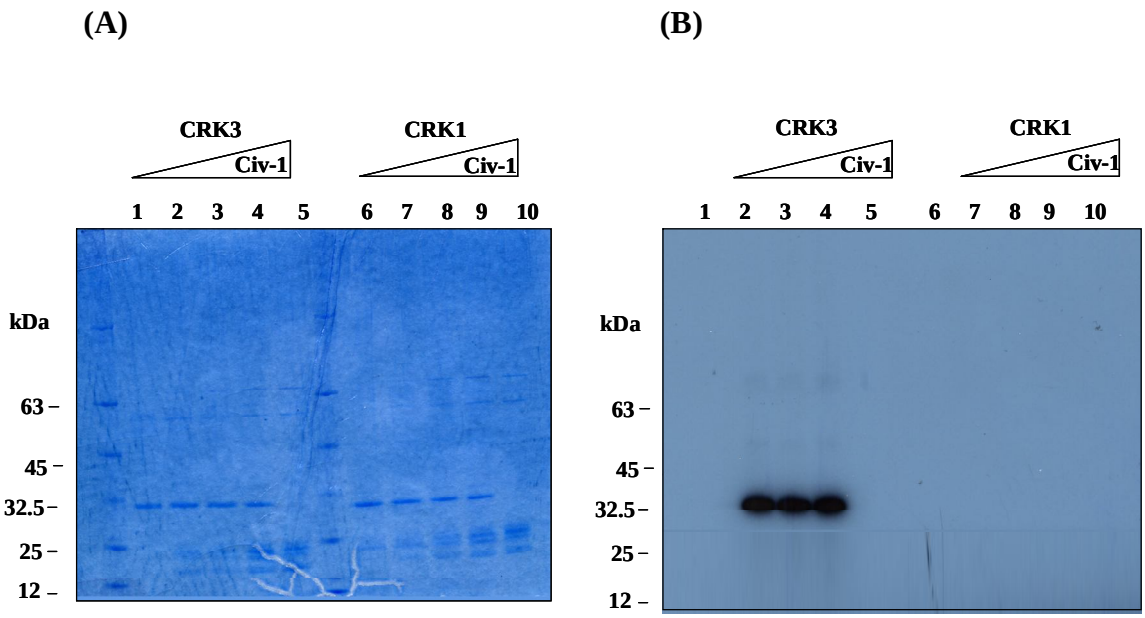


Figure 4.19 Kinase assay using CRK1 as a substrate for Civ1. (A)Coomassie stained gel and (B) the overnight exposed film. LmCRK3 was used as a control on lanes 1 to 4 and CRK1 on lanes 6-9 as a substrate for Civ1. Lanes 5 and 10 contained no CRK and 2.5µg of Civ1. Lanes 1 and 6 contained no Civ1 and 2µg of CRK3 and CRK6 respectively. On lanes 2 to 4 and 7 to 9 Civ1 was added at 1, 1.5 and 2µg while amounts of CRKs were constant at 2µg.

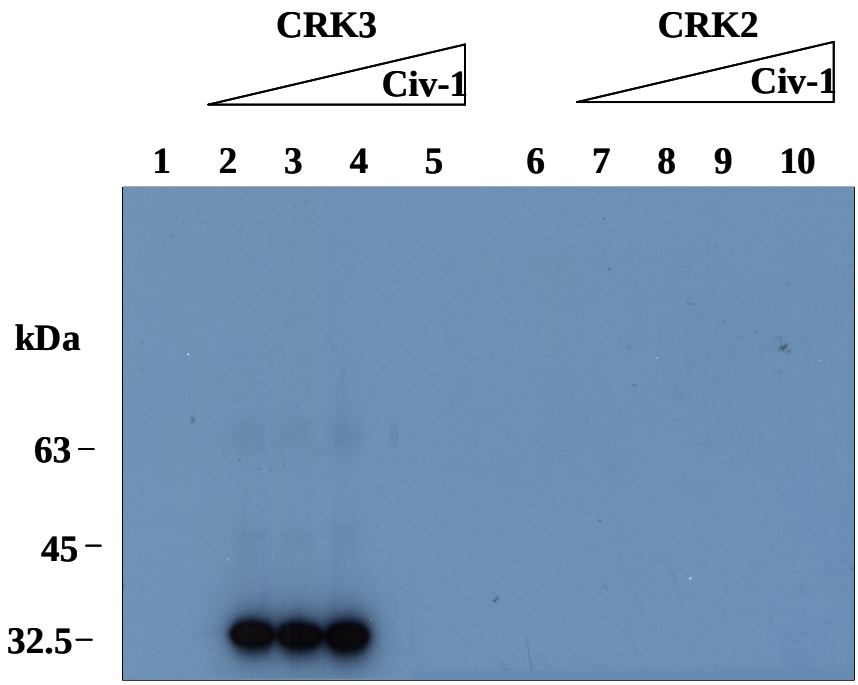


Figure 4.20 Kinase assay using CRK2 as a substrate for Civ1. (A)Coomassie stained gel and (B) the overnight exposed film. LmCRK3 was used as a control on lanes 1 to 4 and CRK2 on lanes 6-9 as a substrate for Civ1. Lanes 5 and 10 contained no CRK and 2.5µg of Civ1. Lanes 1 and 6 contained no Civ1 and 2µg of CRK3 and CRK2 respectively. On lanes 2 to 4 and 7 to 9 Civ1 was added at 1, 1.5 and 2µg, while amounts of CRKs were constant at 2µg.

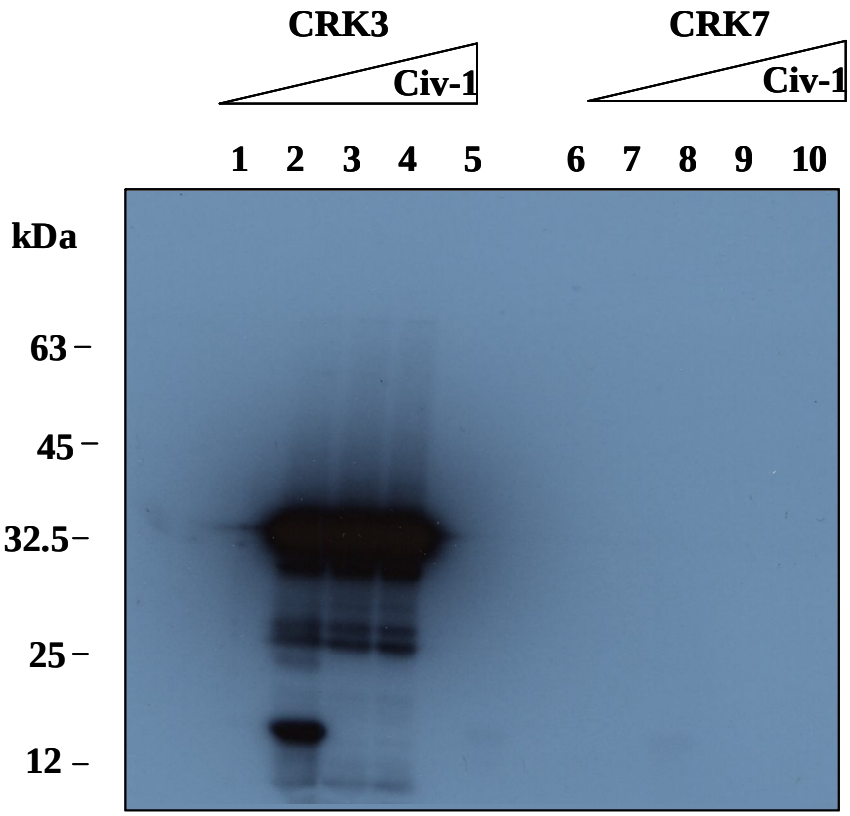


Figure 4.21 Kinase assay using CRK7 as a substrate for Civ1. (A) Coomassie stained gel and (B) the overnight exposed film. LmCRK3 was used as a control on lanes 1 to 4 and CRK7 on lanes 6-9 as a substrate for Civ1. Lanes 5 and 10 contained no CRK and 2.5µg of Civ1. Lanes 1 and 6 contained no Civ1 and 2µg of CRK3 and CRK7 respectively. On lanes 2 to 4 and 7 to 9 Civ1 was added at 1, 1.5 and 2µg while amounts of CRKs were constant at 2µg.

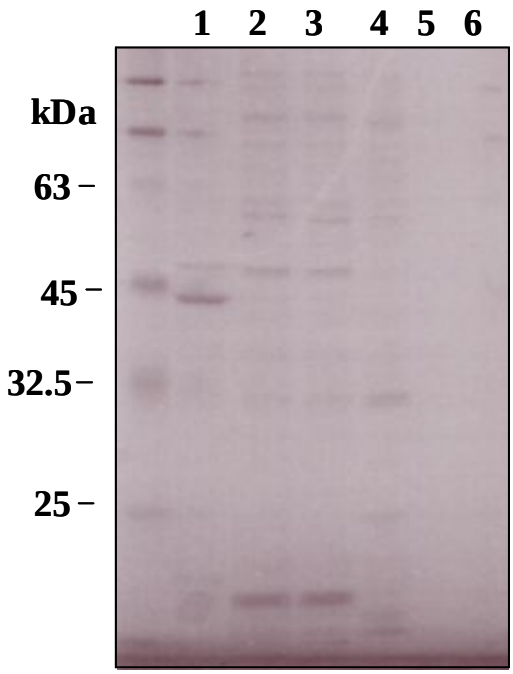


Figure 4.22 Co-purification of CRK2/CYC7his complex. An attempt to purify this complex was carried out. Lane 1, the insoluble fraction; lane 2, the soluble total extract; lane 3 the flow through; lane 4 the column wash and lanes 5 and 6 the elutions.

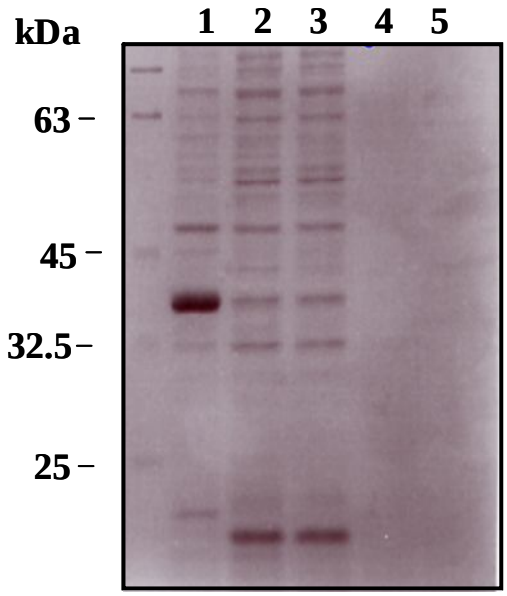


Figure 4.23 Co-purification of CRK1/CYCAhis complex. An attempt to purify this complex was carried out. Lane 1, the insoluble fraction; lane 2, the soluble total extract; lane 3 the flow through; lane 4 the column wash and lanes 5 and 6 the elutions.

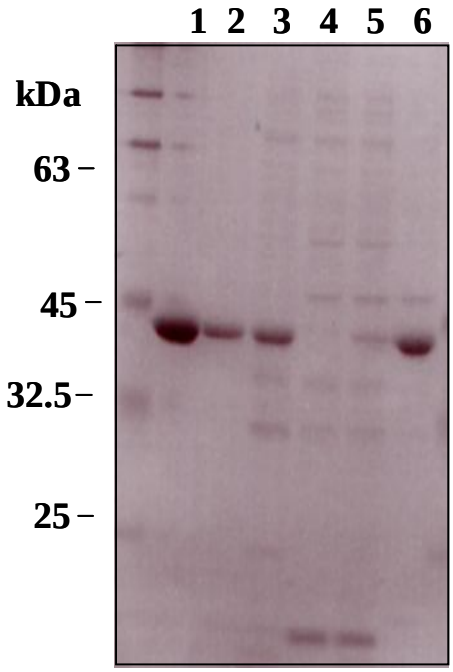


Figure 4.24 Co-purification of CRK1/CYC7his complex. An attempt to purify this complex was carried out. Lane 1, the insoluble fraction; lane 2, the soluble total extract; lane 3 the flow through; lane 4 the column wash and lanes 5 and 6 the elutions.

Table 4.2 Co-expression of cyclin-kinase pairs in *E. coli.* Summary of co-expressions used to attempt the purification of new cyclin:kinase complexes.

	pGL1334	pGL1218	pGL1335	pGL1336	pGL1337	pGL1350	pGL630
pGL1338	**CYCA his/CRK1**	**CYC6 his/CRK1**	**CYC7 his/CRK1**	**CYC9 his/CRK1**	**CYC10 his/CRK1**	**CYC3 his/CRK1**	***Mex*CYCA his/CRK1**
pGL1339	**CYCA his/CRK2**	**CYC6 his/CRK2**	**CYC7 his/CRK2**	**CYC9 his/CRK2**	**CYC10 his/CRK2**	**CYC3 his/CRK2**	***Mex*CYCA his/CRK2**
pGL1340	**CYCA his/CRK3**	**CYC6 his/CRK3**	**CYC7 his/CRK3**	**CYC9 his/CRK3**	**CYC10 his/CRK3**	**CYC3 his/CRK3**	***Mex*CYCA his/CRK3**
pGL1616	**CYCA his/CRK4**	**CYC6 his/CRK4**	**CYC7 his/CRK4**	**CYC9 his/CRK4**	**CYC10 his/CRK4**	**CYC3 his/CRK4**	***Mex*CYCA his/CRK4**
pGL1341	**CYCA his/CRK6**	**CYC6 his/CRK6**	**CYC7 his/CRK6**	**CYC9 his/CRK6**	**CYC10 his/CRK6**	**CYC3 his/CRK6**	***Mex*CYCA his/CRK6**
pGL1349	**CYCA his/CRK7**	**CYC6 his/CRK7**	**CYC7 his/CRK7**	**CYC9 his/CRK7**	**CYC10 his/CRK7**	**CYC3 his/CRK7**	***Mex*CYCA his/CRK7**
pGL1342	**CYCA his/CRK8**	**CYC6 his/CRK8**	**CYC7 his/CRK8**	**CYC9 his/CRK8**	**CYC10 his/CRK8**	**CYC3 his/CRK8**	***Mex*CYCA his/CRK8**

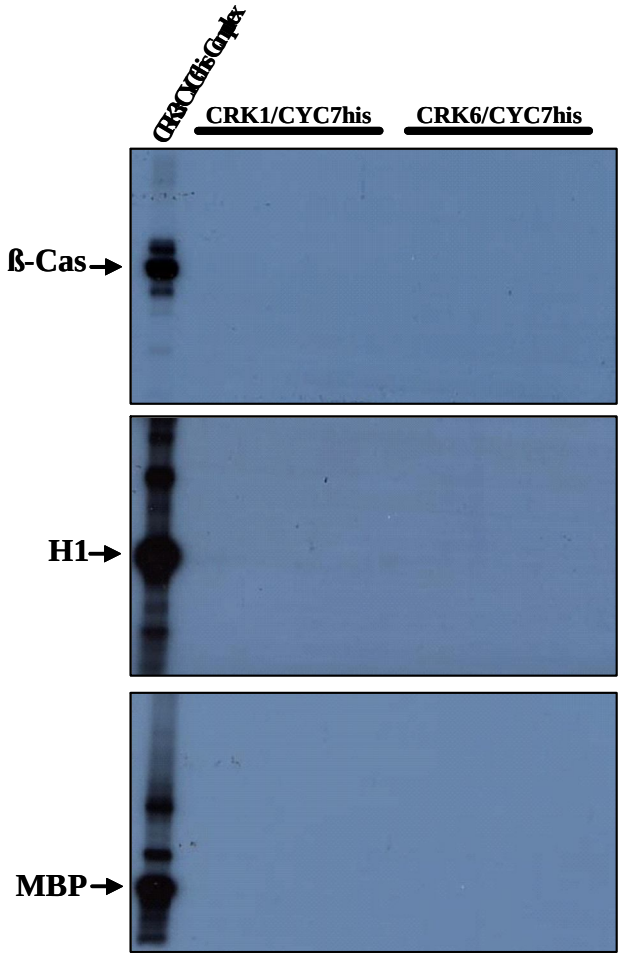


Figure 4.25 Kinase assays with Co-eluted complexes. Elutions from the co-expression purifications were assayed in *in vitro* kinase assays using three different substrates Histone H1, β-Casein and MBP. Reactions were performed in kinase assay buffer containing 5µg of histone H1 or β-Casein or MBP as substrate and γ-ATP labelled with P^{32}. The reaction was incubated at 30^{o}C for 30 min. The reactions were then stopped by adding 2X protein loading buffer. The samples were then loaded in a 12% SDS-PAGE gel, stained with Coomassie, dried and exposed to a ^{32}P particle emission sensitive film. The gel was exposed over night to a general purpose Kodak film. As a positive control, the active CRK3:CYC6his complex was used.

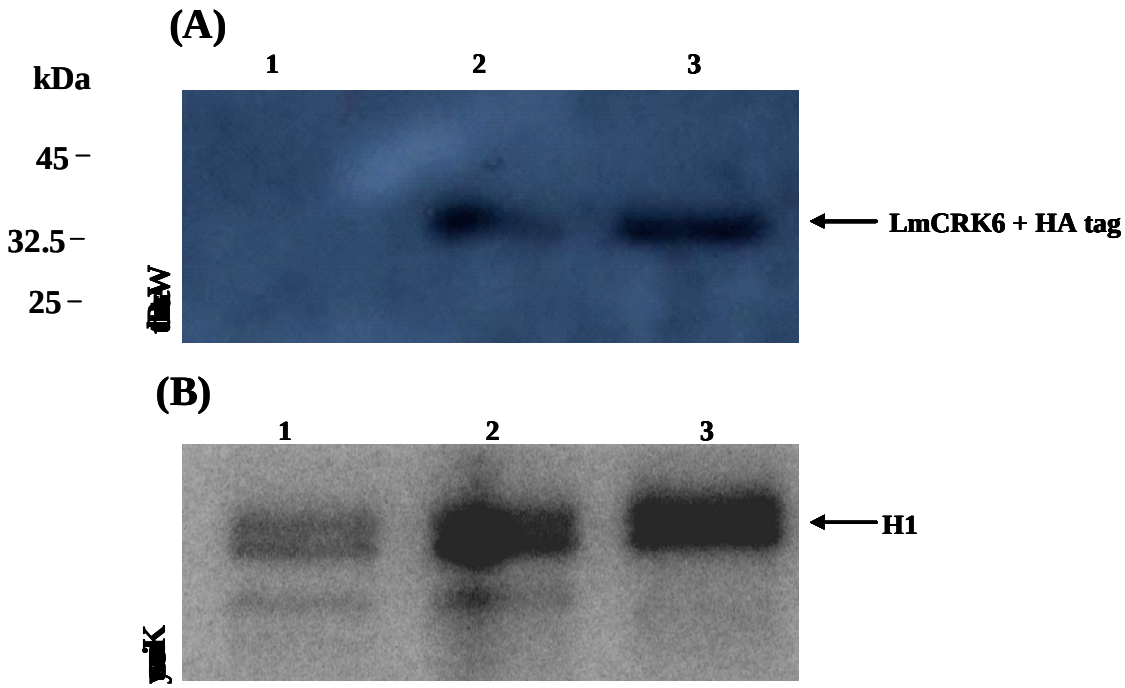


Figure 4.26 Western blot and kinase assay of *in vivo* expressed and purified LmCRK6HA (HA-tagged). (A) Affinity purified CRK6 from overexpressing *L. major* cell lines is detected with HA specific antibody (lane 2). Affinity purified CRK6 from *L. major* cell lines expressing HA tagged CRK6 from a genomic integration in the rRNA locus was also detected with HA antibody (3A). Purification using the same affinity column was performed with WT cells and no protein was detected by the antibody (1A). These purifications were assayed for kinase activity (B). No histone H1 phosphorylation is observed in the WT (1B) elution while both CRK6 HA tagged cell lines possess kinase activity towards histone H1 (2B, overexpressed and 3B, integrated).

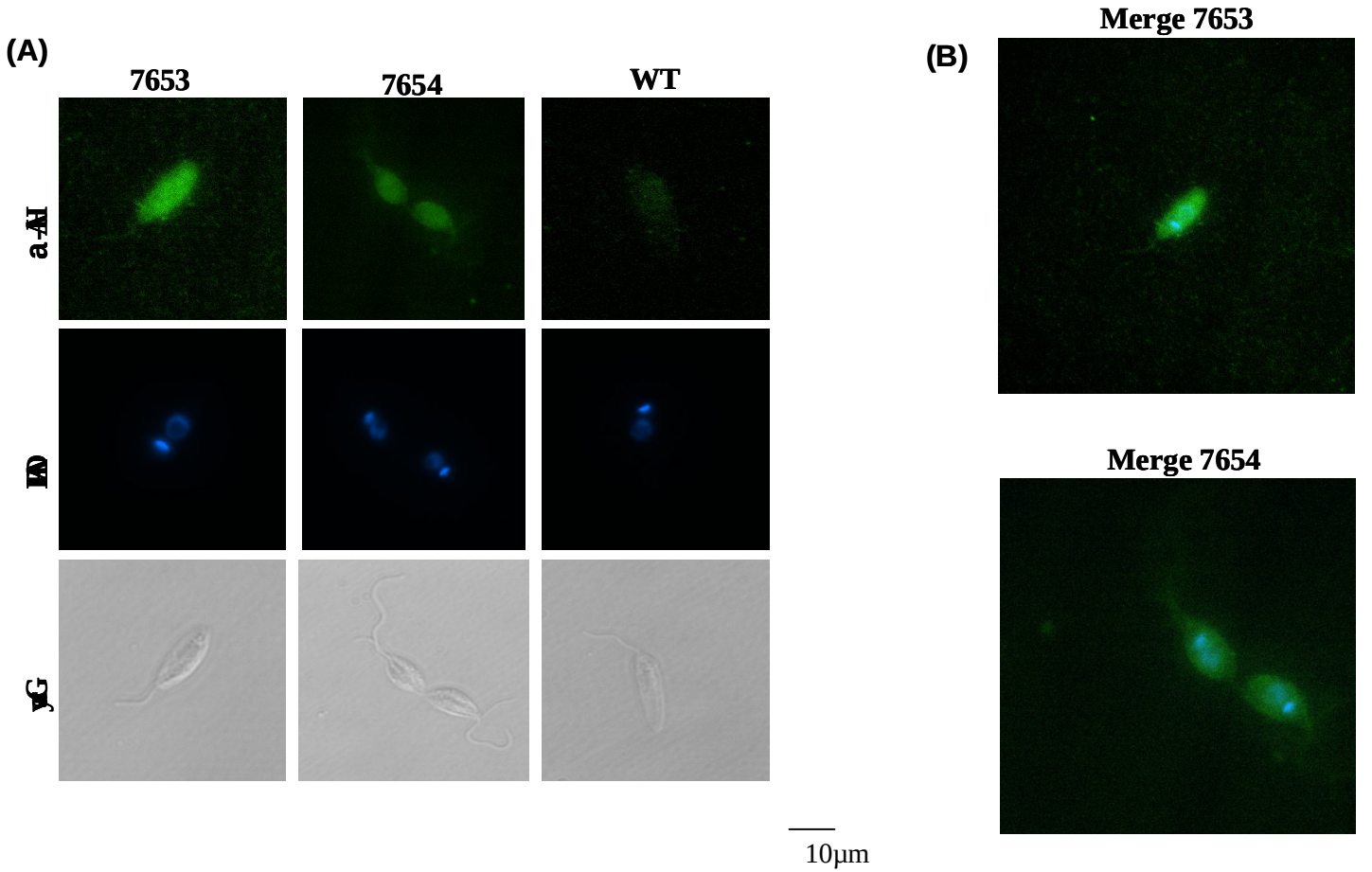


Figure 4.27 Immunofluorescense of *L. major* cell lines expressing HA tagged LmCRK6. On (A), α-HA, DAPI and greyscale can be seen. On (B), merged α-HA and DAPI are shown for LmCRK6HA expressing cell lines. The primary antibody was diluted at 1 in 500 and incubated for 1 hour at room temperature or alternatively overnight at 4°C. The cells were then washed 3 times with 1 ml of PBS. The secondary antibody was diluted at 1 in 1,000 and incubated in the dark for 1 hour at room temperature.

4.3 Discussion

Cyclins regulate the cell cycle by binding to and activating cyclin-dependent kinases. Phosphorylation of specific targets by cyclin-Cdk complexes sets in motion different processes that drive the cell cycle in a timely manner. In yeast, a single Cdk is activated by multiple cyclins to drive the cell cycle. In mammalian cells 11 CDKs are known (Parsons *et al.*, 2005). In trypanosomatids there are also a large number of CRKs and cyclins. While in yeast and mammalian many cyclin:kinase pairs have been identified, in trypanosomatids these interactions are still largely unknown. The ability of these cyclins to target specific proteins and to initiate different cell-cycle events might, in some cases, reflect the timing of the expression of the cyclins; in others, it might reflect intrinsic properties of the cyclins that render them better suited to target particular proteins. Cyclin:kinase pairs have been identified in many different organisms using different strategies (Bloom and Cross, 2007). In *T. brucei* the cyclin E1 partners were assigned *via* complex formation with different CRKs (Gourguechon *et al.*, 2007).

In the current chapter, it was shown that His tagged CYC6 can be used to co-purify non tagged CRK3 from *E. coli* lysates in the form of a cyclin kinase complex (R. Walker, unpublished and see Figure 4.11). The complex was shown to be active against Histone H1, MBP and β-Casein as substrates (Figure 4.25). Other His tagged CRKs from *L. major* were then purified successfully and assayed *in vitro*. It was first tested whether these kinases possess activity as monomers against Histone H1, MBP and β-Casein or have auto phosphorylation capability. It was observed that none of the CRKs tested (CRK1-4 and CRK6-8) had auto phosphorylation capacity or activity as monomers towards the substrates assayed. It was then tested if Civ1 had activity towards CRK1, CRK2, CRK6 and CRK8 kinases, as they all contained a conserved threonine residue at the activation loop (Figures 4.19-4.21). The ability of Civ1 to phosphorylate the serine residue of CRK4 and CRK7 was also tested, since they replace the threonine in these proteins and could

potentially behave like an activation residue. Except for *L. major* CRK3, none of the CRKs tested were phosphorylated by Civ1. These data confirmed the findings that CRK3 is a substrate for yeast Civ1 and provides some evidence that the T-loop region of CRK3 is similar to the Cdc2 T-loop. CRK3 indeed has been proposed to be the functional homologue of this protein (Grant *et al.*, 1998;Hassan *et al.*, 2001). The other CRKs assayed that could not be phosphorylated by Civ1 may require a different kinase capable of recognizing their T-loop or their residues are not suitable for Civ1.

The fact that none of the CRKs tested had monomeric activity towards the substrates used was expected since all CDKs homologues in humans depend upon, not only the cyclin partner, but the phosphorylation of the threonine residue at the activation loop. Recently, it has been shown that human CDK2 can autophosphorylate its T160 residue (Kaldis *et al.*, 1996;Abbas et al., 2007). As it was observed from the experiments in this chapter, none of the CRKs tested as monomers can auto phosphorylate like the human CDK2. It may also be the case that a cyclin is needed to bind and modify structurally the kinase to expose T-loop of other CRKs but not of CRK3. The use of a *Leishmania* CDK7 or Civ1 homologue was not attempted, because no similar protein could be unambiguously be identified in the Leishmania genome.

Taken that LmCYC3, LmCYC7 and CYC10 were insoluble (Figure 4.9) and not suitable for purification and assays, *L. major* CYC9 was then purified (Figure 4.10) and used to try to activate other *L. major* CRKs. This cyclin, as it was shown in the sequence alignments and the phylogenetic tree is the closest cyclin in the *Leishmania* genome to resemble a transcriptional cyclin (C or H). Cyclin H is the human cyclin that activates CDK7/MAT1, the kinase activating kinase that phosphorylates the Threonine residue at the activation loop of mammalian cells (Thuret *et al.*, 1996;Kaldis *et al.*, 1996). None of the purified CRKs from *L. major* could be activated by CYC9. It is unknown if *Leishmania* possesses a single monomeric CAK like yeast or a tripartite complex like humans. But since no clear

homologues from CDK7/MAT1/Cyclin H or CAK can be identified in the genome databases from the *Leishmania* or *Trypanosoma*, CYC9 had to be tested against all possible CRKs available. The lack of activity from the CRKs when CYC9 was added may be due to the wrong choice of substrates used, or CYC9 may activate other kinases not tested, or it may require a third protein, MAT1-like, yet to be identified.

In the present chapter the identification of cyclin kinase activating partners was attempted using an *E. coli* based system. Cyclin genes were cloned in pET15b vectors to express these proteins with a histidine tag. The kinase genes were cloned in a different expression vector pACYC Duet to express the kinases free from any histidine tag. It is important to note that pET plasmids and pACYC plasmids have different origins of replications and therefore are compatible for co-existing inside the same cell. They are suitable for co-transformation and co-expression also because they possess different antibiotic resistance markers. Plasmids from the family pET15 are ampicillin resistant and pACYC are chloramphenicol resistant. Despite the fact that a low expression yield was observed for several of the cyclins tested before, it was expected that if these proteins could bind to kinase subunit as they are synthesised, they would stay soluble. This was already true for the purification of CRK3:CYC6 complex (Figure 4.11).

To explain why no cyclin:kinase complexes could be purified from the co-transformed and induced cell lines it was postulated that pACYC Duet plasmid was expressing the kinases at a low level or not expressing the kinase at all. Although kinases could be observed in some of the purifications, the corresponding band for the histidine-tagged cyclin was not seen (Figure 4.22-4.24). The elution from several of these purifications was tested for kinase activity using three different substrates, histone H1, β-casein and MBP (Figure 4.27). No kinase activity could be detected. To explain this result it was postulated that no pair of cyclin kinase was present in the elution or no appropriated substrate was used.

The immunoprecipation of HA tagged LmCRK6 was successful since kinase activity could be detected in the transfected cell lines and not in the wild type cells control (Figure 4.26). This purification took place after confirmation that an HA tagged protein sized around 36 kDa was detected in the western blot of cell lines S-100 lysates. This result suggests that CRK6 is active in *L. major* but its role in the cell cycle remains unknown. The role of T. brucei CRK6 in the cell cycle was analysed using RNAi and it was postulated that CRK6 may play either a minor role or no role at all (Tu *et al.*, 2005). It is unknown if a cyclin subunit is activating the active purified HA LmCRK6, however, it is likely as the monomeric recombinant CRK6 did not have kinase activity against the same substrate. Further purifications could be used to co-purify partners that interact with CRK6. This approach could be used not only to identify cyclins, but also kinase inhibitors and accessory proteins.

The immunofluorescense assays of the cell lines expressing LmCRK6HA to localize this protein in *Leishmania* cells was carried out. For both transfected cell lines expressing HA tagged LmCRK6 it was observed that this protein appears to be present throughout the cytoplasm of the cells (Figure 4.27). No immunofluorescense signal was detected in wild type cells. Whilst indicative of a cytoplasmic localisation, conformation of the location would be best achieved with mono-specific antibodies raised to recombinant LmCRK6. In interphasic cells Cdk1 is localized in the cytoplasm in three forms: soluble, microtubule associated, or centrosome associated. In late prophase, most of the Cdk1 is rapidly transported into the nucleus (Takizawa and Morgan, 2000). Other Cdks known to be shifted to the nucleos are Cdk2 and Cdk4, they bind bound Cip/KIP molecules for transportation (LaBaer *et al.*, 1997). In the presented experiments LmCRK6 is present in the cytoplasm of the cells in similar manner observed for other important cell cycle related Cdks. Experiments of cell cycle synchronization can be used to determine if CRK6 is driven to the nucleos. However, *L. major* cell cycle synchonizing procedures have not been successful.

Chapter 5

In vivo study of *L. major* CYCA

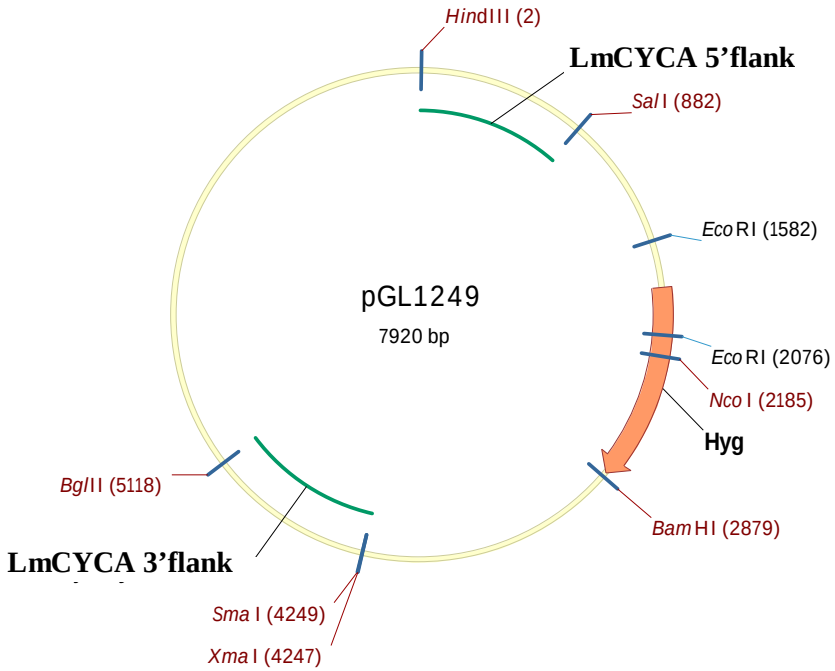
5.1 Introduction

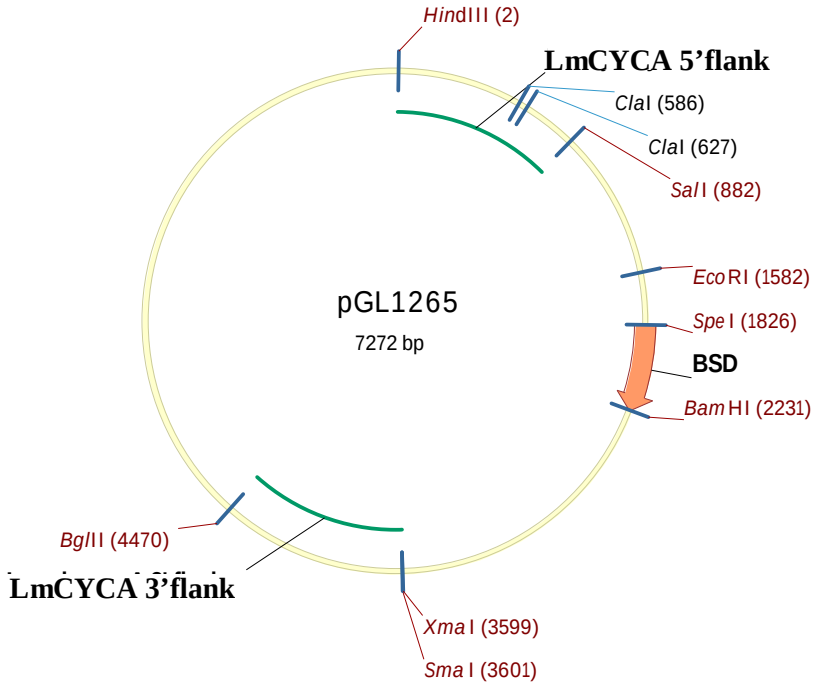
Genome sequence analysis of *L. major* has allowed the study of the cyclin gene family in this organism. It was found that there are 11 cyclin genes, *CYC2-11* and *CYCA*. Ten of these genes (*CYC2-11*) have homologues in *T. brucei*, while *CYCA* is a gene found exclusively in the *Leishmania* genus. Further analysis of the *CYCA* gene has shown that the encoded protein has the highest level of sequence alignment with mitotic cyclinsl, assessed by sequence alignment (Figure 3.3). In this phylogenetic tree CYCA clusters with human mitotic cyclin B-type and dual cyclin A. The other known mitotic cyclin in *Leishmania* is CYC6. However, little is known about this mitotic cyclin. In *T. brucei*, it has been shown that CYC6 does have a role in the cell cycle and interacts with CRK3 to regulate the transition from G2 to M phase (Hammarton *et al.*, 2004). The *in vivo* partner for CRK3 has yet to be identified in *L. major*.

In Chapter 3, I showed that *L. mexicana* and *L. major* CYCA interact and activate *L. major, L. mexicana* and *T. brucei* CRK3 *in vitro*. Based on the aforementioned findings, the aim of this chapter was to identify *in vivo* CRKs with affinity to CYCA in *L. major* cells to confirm the *in vitro* results already obtained (Chapter 3). Furthermore, the study aimed to assess the role of this unique cyclin using gene knock out and *in vivo* tags.

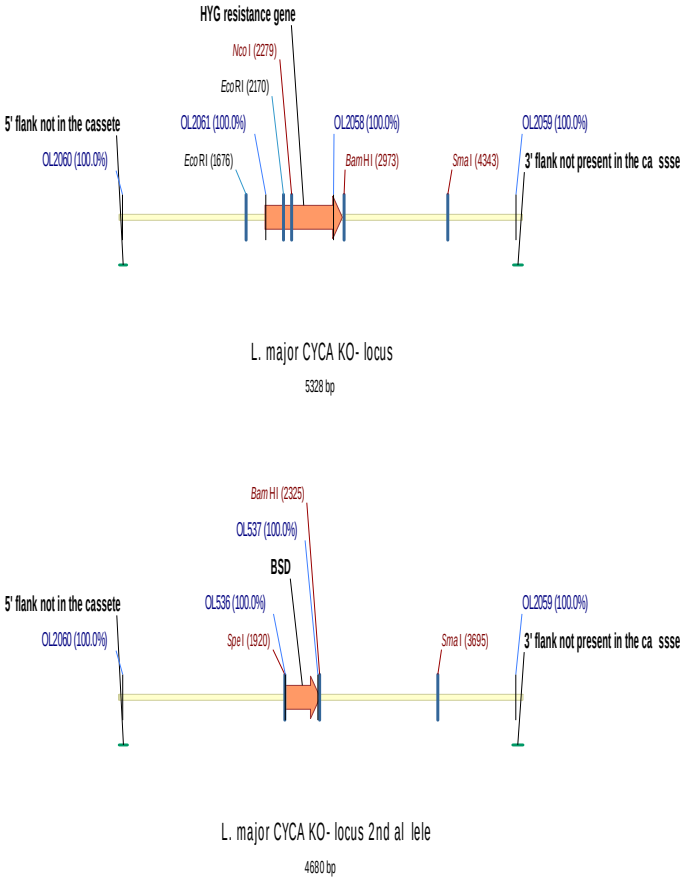
The plasmid pX63 has been used as a backbone to disrupt genes in *Leishmania* (Souza et al., 1994). A derived version of this plasmid pGL102 was used to attempt the disruption of the *CYCA* gene from *L. major*. The expression cassette of the drug resistance gene was flanked at both ends by *CYCA* flanking DNA. This permitted subsequent excision of the entire insert free of vector DNA for homologous recombination with chromosomal *CYCA* sequence (Figure 5.1).

(A)


HindIII (2)
LmCYCA 5'flank
SalI (882)
EcoRI (1582)
pGL1249
7920 bp
EcoRI (2076)
NcoI (2185)
Hyg
BglII (5118)
BamHI (2879)
LmCYCA 3'flank
SmaI (4249)
XmaI (4247)


HindIII (2)
LmCYCA 5'flank
ClaI (586)
ClaI (627)
SalI (882)
EcoRI (1582)
pGL1265
7272 bp
SpeI (1826)
BSD
BamHI (2231)
BglII (4470)
LmCYCA 3'flank
XmaI (3599)
SmaI (3601)

(B)



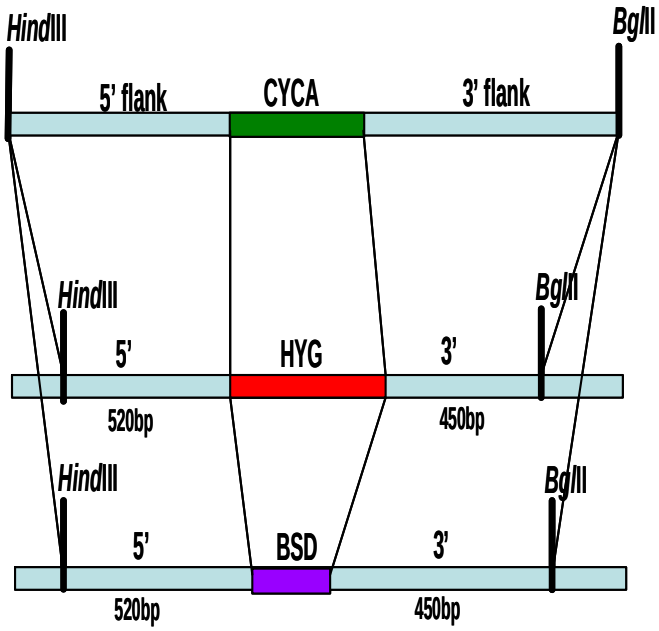


Figure 5.1 (A) Plasmid maps of pGL1249 and pGL1265. These plasmids were linearized by digestion with *Hind*III and *Bgl*II and were used to transfect *L. major* cells in an attempt to knock out *CYCA*. (B) Intergrated CYCA locus with pGL1249 (top, left) and pGL1265 (bottom). Primers used to check for correct integration are also shown in the Figure. PCR details are described in m ethods. Targeted gene disruption of *CYCA* (Right). A representation of the CYCA locus and integrated targeting constructs. Changes in the size of the *Hind*III restriction fragment upon integration of either the CYCA-HYG or CYCA-BSD gene disruption cassettes are shown.

The Tandem Affinity Purification (TAP) method involves the fusion of the TAP tag to the target protein and the introduction of the construct into the host cell or organism. The *in vivo* expressed protein can be purified from cell extracts. Proteins that are associated with the TAP-tagged target are recovered by two step specific affinity purification/elution. The TAP tag consists of two IgG binding domains of *Staphylococcus aureus* protein A (ProtA) and a Calmodulin binding peptide (CBP) separated by a TEV protease cleavage site (Puig et al., 2001).

The HA epitope has been in use for many years (Field *et al.*, 1988). Commercial antibodies for immunoblot and immunofluorescense are available commercially. The HA tag is a small peptide from the haemaglutinin protein of the influenza virus (HA1). The antibodies against HA epitope are also used to purify proteins fused to it. This strategy was used to tag CYCA and CRK6 proteins from *L. major*.

5.2 Results

5.2.1 L. major CYCA knock out attempts

L. major gDNA was used to amplify the flanking regions of LmCYCA and construct two plasmids that were used to attempt to delete the *CYCA* gene. By homologous recombination, attempts to replace the two alleles of LmCYCA with a drug resistance gene present in the plasmids were carried out. The plasmids that were designed and used are shown in Figure 5.1, pGL1249 and pGL1265 have the same 3' and 5' flanking regions but different drug markers that allowed the knock out of the two gene alleles. These plasmids were digested with *Hin*dIII and *Bgl*II restriction enzymes and the gel purified cassettes were used in transfections. However, no first allele knock out of *CYCA* was obtained. The plasmids were sequenced again and this confirmed their sequence. An alternative transfection protocol was used (Robinson *et al.*, 2003) but no integration was obtained

after several attempts. Resistant cell lines were selected for both antibiotics used in the plasmids, but PCR using gDNA from these cell lines showed that no correct integration was obtained. The PCRs used OL2060/OL2061 and OL2058/OL2059 for analysis of the recombined locus containing Hygromycin and OL2060/OL536 and OL2059/OL537 for the analysis of the Blasticidin recombined locus (Figure 5.1). PCR fragments corresponding to the correctly integrated locus were not detected. The strategy is outlined in Figure 5.1.

5.2.2 L. major CYCA TAP tags attempts

L. major CYCA was cloned into pGL955 (derived from plasmid pX53) and fused to a TAP TAG at the C-terminal end of the protein generating pGL1250. This plasmid was used in several attempts to transfect wild type *L. major* cells. Resistant cell lines were obtained, grown and cell lysates were tested by western blot to detect of the TAP tagged CYCA. Two antibodies were used to try to detect the CYCA TAP tagged fusion; anti protein A (Sigma) and anti Calmodulin (Santa Cruz) antibodies. These antibodies failed to detect the TAP tag expression in *Leishmania* cell lysates.

5.2.3 L. major CYCA HA TAG

L. major CYCA was also amplified with a C- or N-terminal HA TAG and cloned into pGL102 (derived from plasmid pXG). The two plasmids generated were named pGL1388, which added an N-terminal HA TAG (HA-CYCA), and pGL1389, which added a C-terminal HA TAG (CYCA-HA) (Figure 5.2). These plasmids derived from pGL102 are episomal vectors that overexpress the proteins in *L. major* WT cells. Cell lines resistant to neomycin were grown and cell lysates were used in western blot analysis using anti HA antibody (Roche). The expression of both C and N-terminal CYCA HA tagged proteins were detected at the predicted size of 35 kDa while no protein was detected in wild type cells (Figure 5.3).

An immune-precipitation (IP) of HA tagged CYCA from *L. major* was performed using HA matrix (Roche). The IP result was analysed on a SDS-PAGE gel stained with silver. A protein corresponding to the expected size of HA tagged CYCA was observed in both HA-CYCA and CYCA-HA immune-precipitates, but not wild type (Figure 5.4). The material bound to the column was probed with an antibody specific to CRK3 and a protein corresponding to CRK3 was observed in HA-CYCA immunprecipitates but not in the CYCA-HA immunprecipitates (Figure 5.5) suggesting that tag may interfere with the ability of the cyclin to bind to the CRK3 when placed at the C-terminus of the protein. The same CRK3 band was not detected in the WT cells. This result showed that CYCA interacts *in vivo* with CRK3 in *L. major* promastigote cells.

The product of the IP was assayed for histone H1 kinase activity. Activity was detected in HA-CYCA and CYCA-HA immune-precipitated samples, but not in WT (Figure 5.6). The transfected cell lines expressing HA-tagged CYCA were used in immunofluorescence in an attempt to localize CYCA in *L. major* procyclic promastigotes. Using anti HA antibody (Roche), both HA-CYCA and CYCA-HA were localized throughout the cell, including the flagellum. Furthermore, it seemed to be present in the flagella pocket region (Figure 5.7).

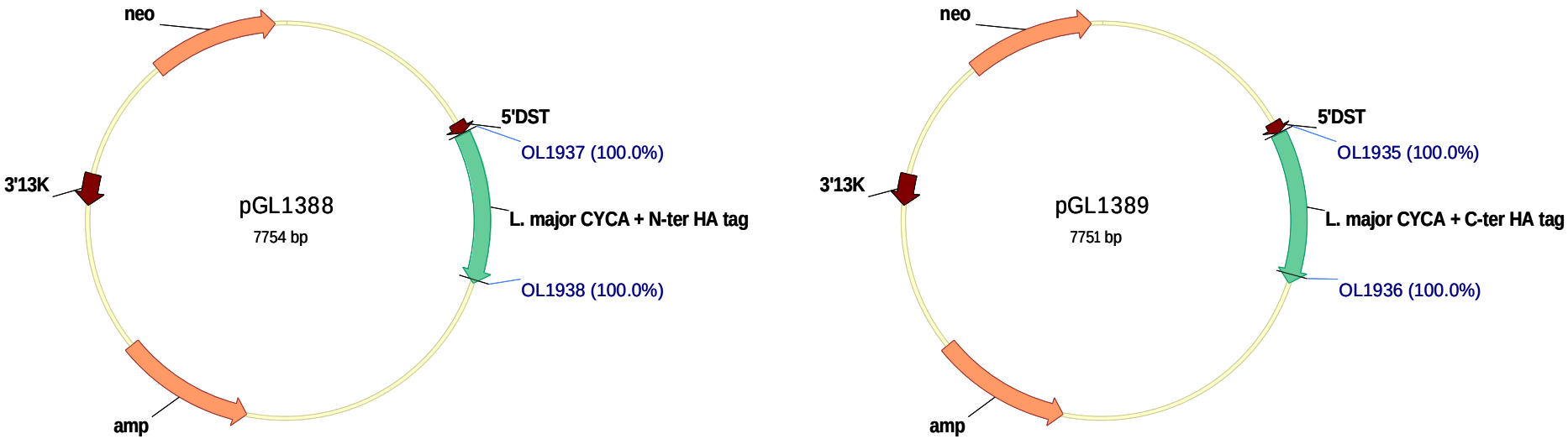


Figure 5.2 Plasmid maps of pGL1388 and pGL1389. These plasmids were used to transfect *L. major* cells in an attempt to over express HA tagged CYCA.

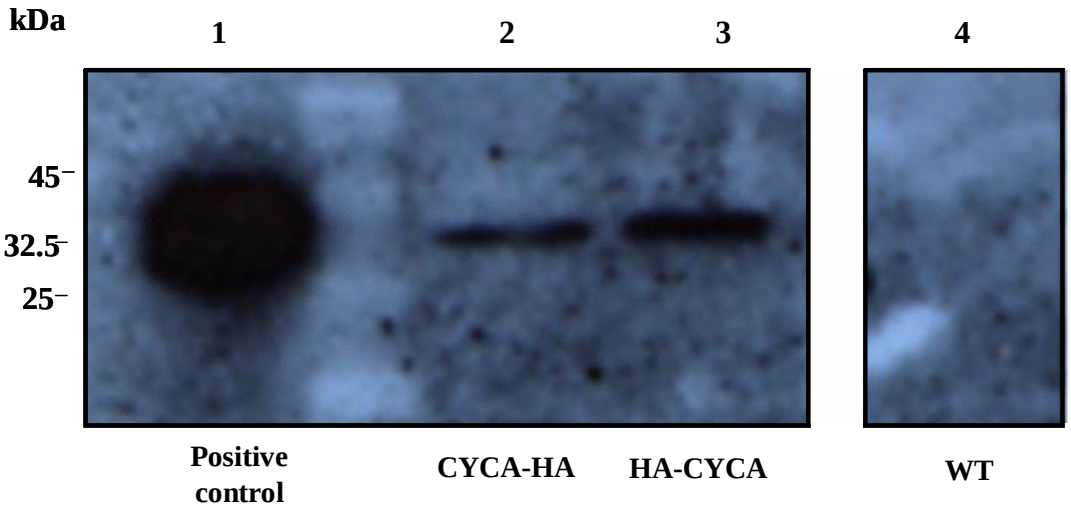


Figure 5.3 Western blot of procyclic promastigote *L. major* cell lysates with anti-HA antibody. Lane 1: 35 kDa HA polypeptide as a positive control, Lane 2: Lysate of *L. major* transfected with CYCA-HA episome, Lane 3: Lysate of *L. major* transfected with HA-CYCA episome, Lane 4: WT cell lysate.

5.3 Discussion

Cell proliferation is regulated by proteins involved in the control of the cell cycle, which is driven by cyclins and their associated kinases. The plasmids generated to transfect *L. major* and knock out the *CYCA* gene were not able to disrupt the gene in the locus. As attempts to knock out *CYCA* in *L. major* failed, the sequences of pGL1249 and pGL1265 plasmids used to transfect *Leishmania* cells were confirmed. Although resistant cell lines were obtained for some of the transfections, PCR analysis of these cell lines showed that plasmid DNA was either integrated in the wrong locus or present in the form of episome DNA, suggesting that CYCA might be an essential gene in *L. major* procyclic promastigote forms

Although this view is now changing, it has been believed that some cyclins and cyclin dependent kinases are nonessential mainly due to redundancy of function (Welcker and Clurman, 2005). In trypanosomes CYC2, CRK1 and CRK3 have been shown to be essential (Mottram et al., 1996b;Grant *et al.*, 1998;van Hellemond *et al.*, 2000;Hammarton *et al.*, 2004). This raised the hypothesis that in these organisms each cyclin-kinase pair might have an independent and essential function in the cell and also it provided further evidence that trypanosomatid CRKs are valid drug targets. In *T. brucei* CRKs as essential proteins for parasite survival has been addressed (Tu *et al.*, 2004;Tu *et al.*, 2005) and shown that in this organism not all CRKs are essential for cell cycle progression and parasite survival. Knocking down CRK genes using RNAi showed that CRK2, 4 and 6 have no additional effect to that of CRK3 silencing. The only CRK that had a complementary effect with CRK3 in these double knockdowns experiments was CRK1 (Tu *et al.*, 2005).

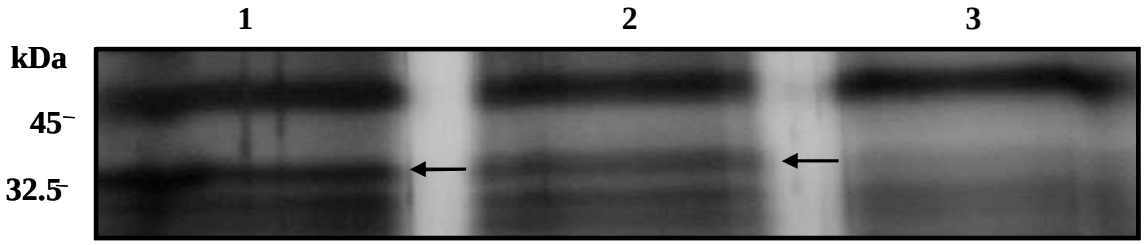


Figure 5.4 Silver stained of SDS-PAGE gel containing eluted materials from the HA antibody affinity purification column. Lane 1: HA-CYCA elution, Lane 2: CYCA-HA elution, Lane 3: WT elution. A protein around 35 kDa, the expected size of HA-tagged CYCA, was detected (arrows).

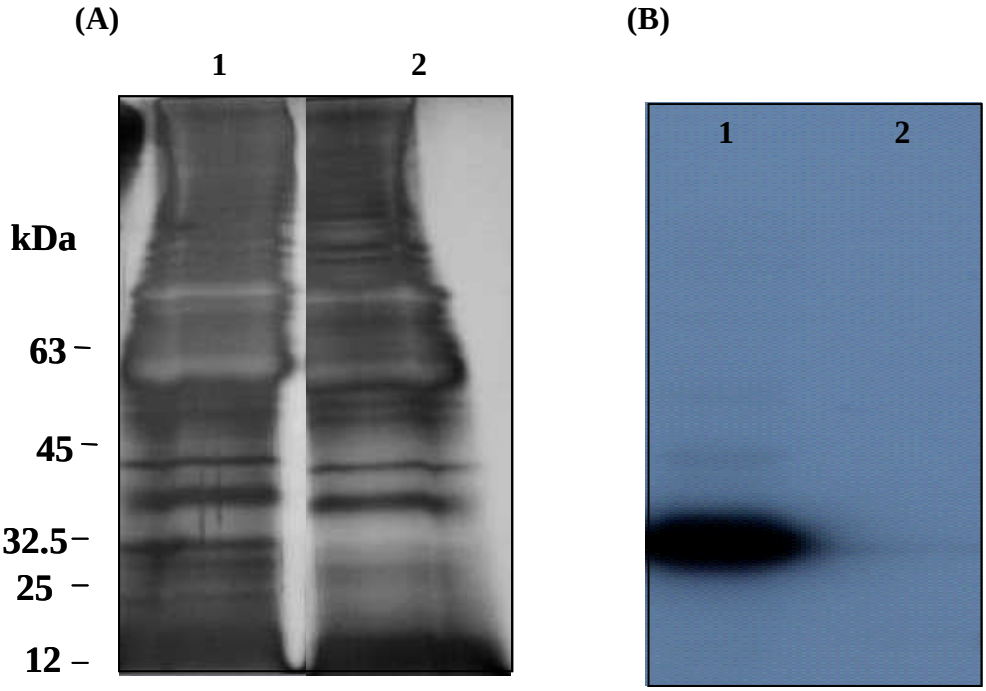


Figure 5.5 (A) Silver stained SDS-PAGE gel of immune-precipitated of HA-tagged CYCA. (B) Western blot of the same samples using anti CRK3 antibody. Lane 1: HA-CYCA immune-precipitation, Lane 2: WT lysate purification. CRK3 was detected only when CYCA was attached to a N-terminal Tag. No CRK3 was detected in the elutions from C-terminal HA tag (not shown).

Some essential genes in *Leishmania* can be disrupted in their first allele while some others can not. Recently published *L. major* metacaspase study is an examples of gene where no single allele knock out can be obtained (Ambit *et al.*, 2007). This is probably because the disruption of just one allele alters the levels of expression and the levels are down regulated in a manner that prevents cell proliferation. Essential genes when disrupted tend to cause changes in chromosome number or ploidy (Cruz *et al.*, 1993). Because disruption of CYCA first allele was not possible it might be that the levels of expression are important for the parasite viability. To test this hypothesis a cell line that expresses CYCA at endogenous levels can be generated before knocking out the genes from the WT locus.

Similarly to the knock out plasmids TAP tagging CYCA plasmid sequences were confirmed and an alternative transfection protocol was implemented (Robinson *et al.*, 2003). Although resistant cell lines were again obtained, the TAP TAG fused protein could not be detected in Western blots. Tagging CYCA with an HA epitope was successful for both C- and N-terminal fusions. Western blots detected the expression of HA-CYCA and CYCA-HA in the lysates of *L. major* promastigotes (Figure 5.3). Based on this finding, purification experiments were carried out using an affinity purification matrix with an attached HA antibody (Roche). It was shown that several proteins interact in a non-specific manner with the resin and very low amounts of CYCA were purified. The identity of the HA-tagged protein detected in the silver stained gels, was confirmed by the western blot with the HA antibody (Figure 5.4). The purified material was used in a kinase assay and both C- and N-terminal HA tags purified showed kinase activity (Figure 5.6). This suggests that the presence of the tag does not prevent interaction of CYCA with the activating kinase. CRK3 was shown to be one of the kinases that interact with HA-CYCA (Figure 5.5).

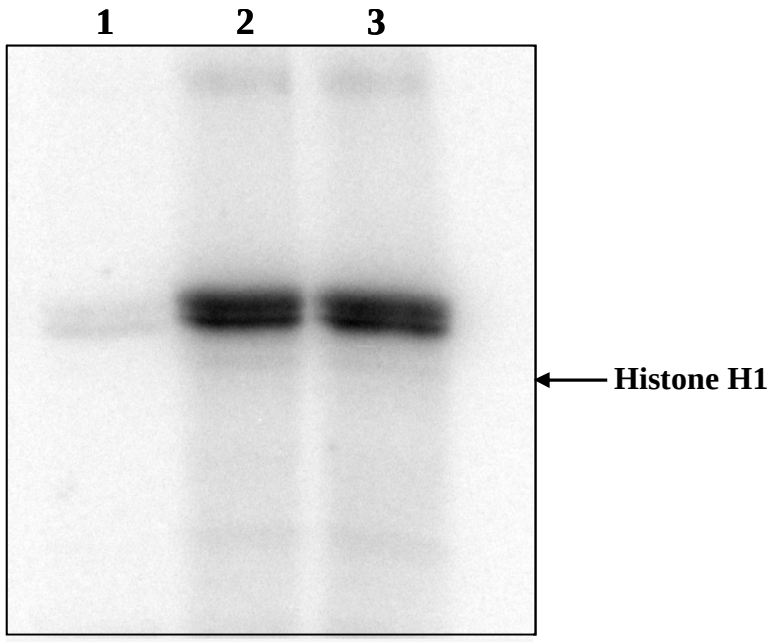


Figure 5.6 *In vitro* Histone H1 kinase assay. Activity of immune-precipitated HA-tagged CYCA was assayed using Histone H1 as substrate. Lane 1: WT *L. major* elution, Lane 2: Elution of *L. major* transfected cell lines expressing CYCA-HA, Lane 3: Elution of *L. major* transfected cell lines expressing HA-CYCA.

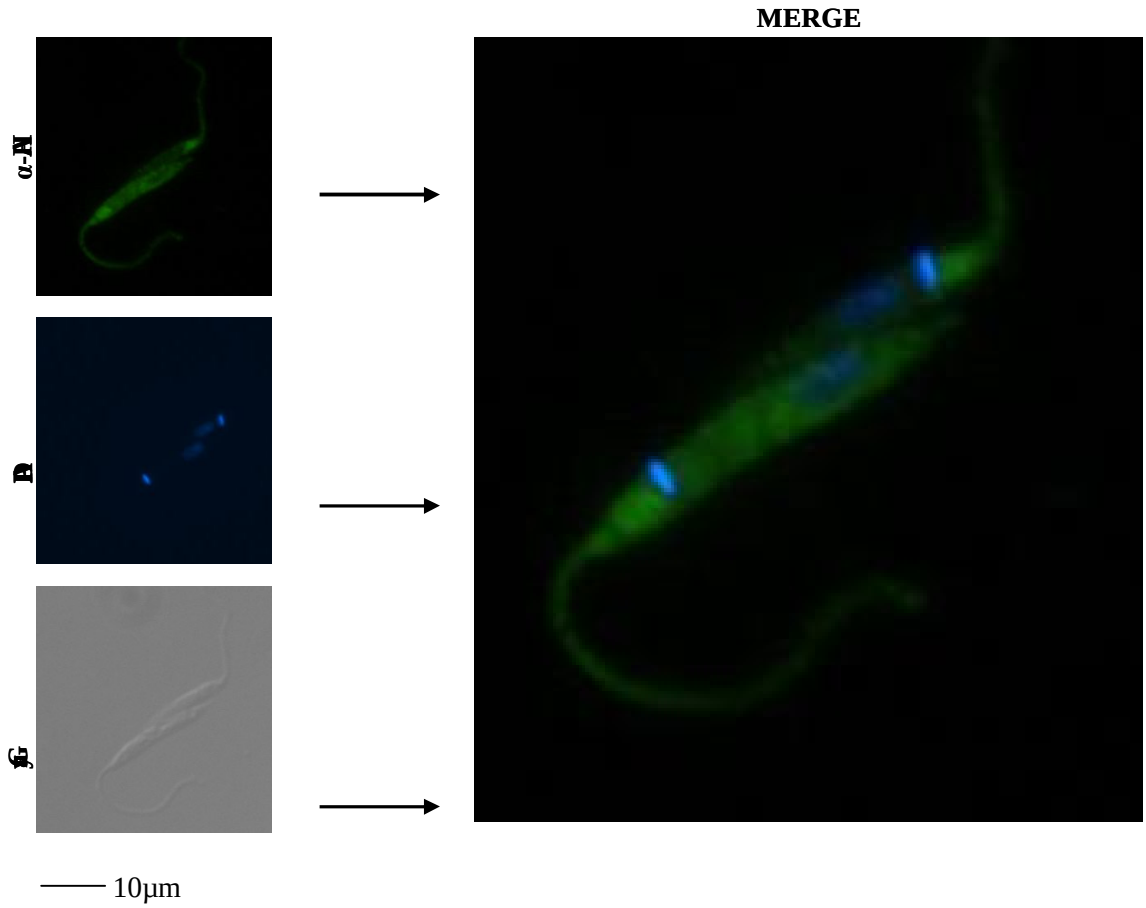


Figure 5.7 Immunofluorescence of *L. major* procyclic promastigotes transfected with HA-CYCA. On the left, α-HA, DAPI and greyscale can be seen. On the right, merged α-HA and DAPI are shown. The primary antibody was diluted at 1 in 500 and incubated for 1 hour at room temperature. The cells were then washed 3 times with 1 ml of PBS. The secondary antibody (α-Mouse FITC conjugated antibody) was diluted at 1 in 1,000 and incubated in the dark for 1 hour at room temperature.

The CRK3 antibody detected CRK3 in the N-terminal CYCA HA purification but not in the C-terminal tagged protein or in the WT cells. This provides evidence that other kinases might be binding CYCA and being activated thus generating kinase activity. Previous *in vitro* and *in vivo* studies have shown that CYCA binds and activates CRK3 in *L. donovani* cells and CYCA is expressed in the S phase suggesting a potential role of CYCA in G1 or S phase of the cell cycle (Banerjee *et al.*, 2006). Given that CYCA might be one of the activating cyclins of CRK3 in all *Leishmania* species, it could be recognized as a potential drug target. CRK3 itself has already been established as a drug target, since it is an essential gene of kinase cyclin dependent that regulates the cell cycle of *L. mexicana* (Grant *et al.*, 1998). However, the idea of using a cyclin inhitors to arrest the cell cycle is recent (Grant *et al.*, 1998;Kozar *et al.*, 2005). The only specific inhibitors of a cyclin is INK4a that acts by binding to the Cdk4/6, altering the structure of the cyclin binding domain and thus preventing the activation of the kinases (Kozar *et al.*, 2005).

Cyclin B binds to the cyclin-dependent kinase, cdk1, to form the cyclin B/cdk1 complex, while cyclin A both alone and in complex with its cognate kinase, cdk2. The cyclin E/cdk2 is also an important functional pair. However, not only Cdks are able to bind multiple cyclins. Cyclins also are able to bind multiple Crks in yeast, mammals and kinetoplastids as well (Gourguechon *et al.*, 2007).

Cyclin D1 accumulates in the nucleus during the G_1 interval and it relocalizes to the cytoplasm during S phase. The essential functions of cyclin D1 require its nuclear localization, and thus the redistribution of cyclin D1 complexes to the cytoplasm following G_1 implies that regulation of cyclin D1 nucleocytoplasmic distribution is necessary for maintaining cellular homeostasis (Benzeno and Diehl, 2004). At M phase, cyclin B1 is phosphorylated in the cytoplasmic retention sequence, which is required for nuclear export. During interphase, cyclin B1 shuttles between the nucleus and the cytoplasm because constitutive nuclear import is counteracted by rapid nuclear export. In M phase, cyclin B

moves rapidly into the nucleus coincident with its phosphorylation, an overall movement that might be caused simply by a decrease in its nuclear export (Hagting *et al.*, 1999).

The localization of individual cyclins to different subcellular compartments has been established, G1 cyclin is primarily nuclear, whereas mitotic cyclins are cytoplasmic and can localize to sites of polarized growth (Miller and Cross, 2000;Bloom *et al.*, 2007). This localization pattern contributes to the abilities of cyclins to regulate different substrates (Bloom *et al.*, 2007). Nuclear export signals are present in mitotic cyclins but they also have a role in the nucleus despite being mainly cytoplasmic (Bloom *et al.*, 2007). The localization of LmCYCA in the cytoplasm does not prevent a role in the nucleus as well since it appears to be distributed all over the cell including nucleus although in small amounts (Figure 5.7). An interesting experiment can be done to determine if CYCA would be transported to the nucleus during the cell cycle progression. Tranfected *L. donovani* could be used to overexpress tagged CYCA in a synchronized cell culture and establish the localization of this protein in 2N2K cells.

When CYCA-HA tagged was probed in immunofluorescense experiments with fixed cells to localize the protein, CYCA was found to be distributed throughout the cell including the flagellum, although there appeared to be a more concentrated signal in the flagellar pocket area of *Leishmania*. As mentioned, CYCA was associated to activities in the S-phase (Banerjee *et al.*, 2006) in *L. donovani*. The presence of CYCA in the flagella pocket may be associated with S-phase events in the cell cycle, since it is known that this organelle is one of the first cellular structures to duplicate (Hammarton *et al.*, 2005). However, CYCA is being over-expressed, so the location may be an artefact of the expression system. To avoid this artefact possibility an endogenous tagged CYCA can be used together with synchronized cell cultures to analyse the cell cycle progression and CYCA localization.

Chapter 6

General Discussion

Leishmaniasis is a disease with multiple clinical manifestations, multiple endemic regions around the world and is caused by multiple *Leishmania* species (Murray *et al.*, 2005a). Around 2 million new cases are considered to occur annually, with an estimated 12 million people presently infected worldwide and an effective vaccine against leishmaniasis being still unavailable (WHO-http://www.who.int/). The results obtained from the experiments presented in this study aimed to further explore the role of cyclin dependent kinases and cyclins in the protozoan parasite *Leishmania major*. Cdks in kinetoplastids, CRKs, are the key regulators that allow cells to progress through different cell cycle phases and promote parasite proliferation during infection. Previous studies have identified CRK3 as the CDK1 orthologue, the cyclin dependent kinase that controls entry into mitosis (Grant *et al.*, 1998;Hassan *et al.*, 2001). However, these studies failed to identify CRK3 cyclin binding partners *in vivo* or *in vitro* and as such studying the biochemistry of the cyclin dependent kinases involved in the regulation of the cell cycle progression in these parasites becomes essential. In chapter 3 of this study, the results presented showed that *L. major* CYCA is capable of activating CRK3 in an *in vitro* kinase assay using histone H1 as substrate. The association of the cyclin subunit to the Cdk not only activates the kinase but also determines the specificity to the substrates. The differences in substrate specificity displayed by the same kinase when bound to different cyclins is determined by electrostatic properties at the substrate-binding pocket known as the cyclin groove (Lee et al., 2007). In this way *L. major* CYCA was responsible for CRK3 specificity towards histone H1 substrate in the *in vitro* kinase assays performed.

The CRK3/CYCA active complex was then used to analyse the effect of the phosphorylation at the CRK3 activation threonine using a kinase activating kinase (yeast CAK or Civ-1). Phosphorylated CRK3 activity was compared to non-phosphorylated CRK3 and it was found that the phosphorylation promotes a 5-fold increase in kinase activity of the complex. The accessory protein Cks1 was assayed *in vitro* with the active CRK3/CYCA complex and it was shown that Cks1 might have an inhibitory effect when

histone H1 substrate is used. Finally, the IC_{50} for two different kinase inhibitors (Flavopiridol and Indirubin) was determined for the *in vitro* CRK3/CYCA complex and compared with the values found for the *in vivo* purified CRK3. Similar values were obtained suggesting that the *in vivo* complex is indeed represented by the recombinant complex. The development of recombinant active cyclin dependent kinases from *Leishmania* is important not only for studying the biochemistry of the complex *in vitro* but also may be useful for the screening of large kinase inhibitor libraries. The high throughput screening of a large number of molecules aims to identify inhibitors that would structurally be able to inhibit the kinase activity of a leishmanial CRK, while not interfering with most host kinases. Kinase inhibitors were first thought to be useful for cancer therapy to prevent uncontrolled cell proliferation. In a similar manner, parasite infection may be prevented using kinase inhibitors specific for essential CRK proteins like CRK3. An increasing number of protein kinases of parasitic protozoa are being evaluated as drug targets, just as they had been in trials to treat a wide range of other diseases and syndromes, such as cancer, cardiovascular disease and Alzheimer's disease (Johnson, 2007). In recent years, pharmaceutical companies have invested heavily in the development of new compounds directed against specific protein kinase targets, and there are a wide range of protein kinase inhibitors that have entered clinical trials (Naula *et al.*, 2005;Johnson, 2007). Finally, it has been shown that inhibition of kinases using specific compounds is possible. In this way, differences in the biochemistry of different kinase complexes would result in different sensitivities to different inhibitors (Thaimattam *et al.*, 2007). This has made possible the development of kinase specific compounds able to block the activity of individual kinases of interest. Human Cdk1 and Leishmanial CRK3 may be responsible for entry into mitosis in both organisms, however, they are very different kinases as our sequence analysis and biochemistry experiments have shown. CRK3 possess only 34% identity with human Cdk1 and phosphorylation of the activation loop does not generate a similar stimulatory effect in the parasite enzyme. Furthermore, the activating subunity of these kinases, the cyclin

partner, is even less conserved. The structure at the ATP binding site contained in the kinase is the major determinant in the inhibitors compounds mode of action. The cyclin have not been implicated in the selection of specific inhibitors but it certainly has an effect in determining the substrate specificity and therefore should have an effect in the final structure of substrate recognition near site and ATP binding pocket.

In the following chapter 4, yeast Civ-1 purified from *E. coli*, was used to try to phosphorylate, in a similar manner, the activation of threonine/serine residues from other *L. major* CRKs. The kinases assessed were CRK1, CRK2, CRK4, CRK6 and CRK7. None of these were phosphorylated by Civ-1 suggesting that the only CRK under this type of regulation is CRK3. A trypanosomatid homologue to Civ-1 or Cdk7 could not be identified by blast analysis using geneDB standard settings. This may be because this corresponding kinase in trypanosomatids have diverged into or is not present and organisms like *Leishmania* do not need to phosphorylate and activate the T-loop. The evidence to support this hypothesis is on the fact that there is already a strong kinase activity detected from CRK3 caused by CYCA binding itself. The increase in kinase activity when Civ-1 is added to the system is only a 5-fold increase.

L. major CRK1-4 and CRK6-8 were tested in kinase assays by mixing under described conditions with *L. major* CYC9 and kinase activities towards three different substrates were assessed. *L. major* CYC9 was not able to activate the above kinases and the kinase subunit that interacts with this cyclin could not be identified. Following the inability of CYC9 to activate the aforementioned kinases, we tested if CYC9 was expressed in the parasite. It was also tested if the expression of cyclins CYCA, CYC2, CYC7, CYC9 and CYC10 was also present. The mRNA for synthesis of all these cyclins was detected, confirming expression of the cyclins in the parasite. However, the binding partner of CYC9 remains unknown. The identification of a cyclin dependent kinase that controls transitions at the S-phase can provide an important drug target since DNA replications is

an essential process for parasite proliferation. In the protein sequence alignments shown in Figure 4.2, CYC9 is the most similar *Leishmania* cyclin to human transcriptional cyclin C and H and although CYCA has been suggested to be involved in S-phase processes (Banerjee *et al.*, 2006), no conclusive evidence for the presence of a transcriptional cyclin in kinetoplastids is available. It is known that some cyclins have the hability to bind multiple Cdks. For instance cyclin A can bind both Cdk1 and Cdk2 to promote mitosis and S-phase respectively (Walker and Maller, 1991). *T. brucei* CYC2 (also named cyclin E1) was shown to interact with several Cdks using yeast two hybrid system (Gourguechon *et al.*, 2007). *T. brucei* CYC6 (also named cyclin B2) has also been shown to interact with CRK3 and control entry into mitosis (Hammarton *et al.*, 2003a). However, it was suggested that CRK1 is the major cyclin dependent kinase regulating transition from G1 to S-phase (Gourguechon *et al.*, 2007). The previous results obtained in this study with CRK3 and CYCA have successfully produced cyclin activated kinase. Although the expression of the mRNA of CYC9 was confirmed (Figure 4.8) and the same methods of expression, purification and kinase assay applied, no active kinase was observed when CYC9 was used.

In chapter 5, the *L. major* CYCA was used to elucidate the characteristics of this cyclin *in vivo*. A gene disruption strategy aimed to replace the two genomic alleles of this protein gene by homologous recombination. Plasmids were developed with flanking regions of this gene placed in association with two different drug resistance genes, one for each of the allele's disruption. These constructs were not able to produce the first allele knock out suggesting that not only this gene might be essential but the levels of expression may also be important. To explain why some resistant cell lines, but no disruption, were obtained in transfections performed with these plasmids, specific PCR for the CYCA locus was performed with genomic DNA extracted from these cells. These PCR reactions showed that the plasmids might be functioning as episomes conferring resistance to the cells but leaving the locus intact. Tagging *L. major* CYCA was also attempted *in vivo* using two

different strategies (i.e. two different tagging systems). The first tag employed was the TAP tag system (Puig *et al.*, 2001). Although drug resistant transfected cell lines were obtained, no tag detection could be observed by western blot using different tag-specific antibodies (α-protein-A and α-calmodulin antibodies). The second tag employed was HA, the 9-amino acid sequence YPYDVPDYA, derived from the human influenza hemagglutinin (HA) protein. Plasmids that contained C and N-terminal HA tagged *L. major* CYCA were used to transfect WT cells and cells extracts of resistant cell lines analysed by western blot. Both C and N-terminal HA tagged CYCA were detected by the α-HA antibody. Following the confirmation of the presence of the tagged CYCA in the cell extracts an affinity purification using an HA affinity matrix was attempted and the matrix binding material was used in *in vitro* kinase assays (Figure 5.6). The presence of kinase activity towards Histone H1 confirmed that CYCA was being successfully immunoprecipitated in complex with a kinase partner. The identity of the co-eluted CRK could be confirmed using specific α-CRK3 antibody that detected CRK3 in the eluted material. However, CRK3 is only observed for the N-terminal CYCA tagged and not at the C-terminal. This suggests that more than one CRK can be activated by CYCA and not only CRK3, since kinase activity is detected for both cell lines expressing the differentially positioned tags.

The *L. major* protein kinase CRK6 is a cyclin dependent kinase that has not yet been studied in detail. In the present study, LmCRK6 was HA tagged, immunopreciptated and tested for kinase activity. LmCRK6 has *in vitro* kinase activity towards Histone H1 when imunoprecipitated from live parasites. This finding confirms that CRK6 is indeed active in the *L. major* procyclic form. In *T. brucei* (Tu *et al.*, 2005), RNAi experiments have shown that CRK6 plays a minor or no role in the cell cycle of these parasites. A cyclin binding partner for CRK6, if any, remains unknown. However, the data presented in the chapter 4 of this study suggests that there might be at least one activation requirement, since no kinase activity was observed using monomeric CRK6. Furthermore, CRK6 threonine

residue could not be phosphorylated by Civ-1. This suggests that the phosphorylation by a CAK like protein might not be required for activity of these CRKs that the enzyme is capable of recognizing this residue is still unknown.

Future plans

Recombinant CRK3:CYCA complex can be used in high throughput screening of kinase inhibitors that are specific for the parasite kinase. This would be important to generate more efficient drugs for the treatment of Leishmanisis. The identification of a kinase activating kinase similar to Civ-1 or Cdk7 was not possible using sequence comparisons, however, cloning and expressing a tagged truncated T-loop region from CRK3 can be used to affinity purify interacting proteins from cell extracts. Another strategy to identify a *Leishmanial* Civ-1 would be to fuse CRKs T-loop region to GST and also use it in an affinity column against cell extracts. Overexpressing the same truncated T-loop region HA tagged *in vivo* can also be attempted with the episomal system tested in this study.

To identify *L. major* CYC9 kinase counterpart an affinity column can be made with his-tagged recombinant CYC9 to affinity purify binding proteins from cell extracts since activation of tested kinases was not successful. The same strategy can be used for the kinase with unknown cyclin partner like CRK1, CRK2, CRK4, CRK6, CRK7 and CRK8.

Cell lines of *L. major* expressing HA tagged CRK6 are already made and can be used to search for a cyclin partner as well as a kinase activating kinase in *Leishmania* through affinity purification in an immobilized column. The HA tagged CYCA cell line can be used to better study the localization of this protein in the cells *in vivo*. It is important that immunofluorescence experiments with a second antibody that targets the flagellar pocket proteins are used to co-localize CYCA with this important organelle. It is also possible that the same constructs are used to transfect *L. donovani* and cell cultures of this *Leishmania*

species are synchronized by Hydroxy Urea and the cell cycle stages where CYCA is active, as well as where in these cells it is detected can be better studied.

Appendix

List of buffers and reagents

Aminolink Quenching Buffer: 1M Tris•HCl, pH 7.4

Aminolink Reduction solution: 50mM Sodium cyanoborohydride (NaCNBH3)

Aminolink wash solution: 1M Nacl

Aminolink Coupling Buffer: 0.1M sodium phosphate, 0.15M NaCl, pH 7.2

Ampicillin: 100mgml^{-1} stock in 50% ethanol. Stored at -20^{o}C.

Blocking solution for Western blots: 5% non-fat dried milk in PBS/0.01% tween 20.

Blocking solution for immunofluorescense slides: 0.1% Triton X-100, 0.1 % BSA.

Chloramphenicol: Stock solution at 34 mgml^{-1} in ethanol. Used at 170 µgml^{-1}.

Coomassie staining solution: 0.25 g of Coomassie Brilliant Blue R-250 in 90 ml of methanol: H_2O (1:1.v/v).

Coomassie destaining solution: 20% Methanol, 5% Acetic Acid in H_2O.

DNA loading buffer: 0.025% bromophenol blue, 0.25% xylene cyanol, 40% sucrose in distilled H2O. Stored at 4^{o}C. Alternatively: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll (Type 400; Pharmacia) in H_2O. Stored at room temperature.

DNase I dilution buffer: 10mM Tris-Cl (pH 7.5), 150mM NaCl, 1mM $MgCl_2$

Eletroporation Buffer (transfection buffer): 21mM HEPES pH 7.5, 137mM NaCl, 5mM KCl, 0.7mM phosphate buffer, 5mM glucose. Stored at 4°C. Alternatively: 120mM KCl, 0.15mM $CaCl_2$, 10mM K_2HPO_4, 25mM HEPES, 2mM EDTA and 2mM $MgCl_2$; pH 7.6.

Ethidium Bromide: 10mgml^{-1} stock in distilled H2O. Stored at room temperature.

GST Elution Buffer: 10mM glutathione, 50mM Tris-HCl, pH 8.0

Ion Exchannge Buffer A: 50mM Tris-HCl, 5mM EDTA pH 7.0.

Ion Exchannge Buffer B: A+ 1M NaCl.

Kanamycin: Stock solution at 10 mgml^{-1} in H2O. Used at 50 µgml^{-1}.

Kinase assay buffer: 50mM MOPS pH 7.2, 20mM $MgCl_2$, 2mM DTT, 10mM EGTA. Stored at 4°C.

Kinase assay mix: 5 µl histone H1 (10mgml^{-1} stock), 8 µl ATP (100µM stock), 186 µl kinase assay buffer, 1 µl γ-^{32}P-ATP (50µCi).

Kinase storage buffer: 20mM HEPES, pH7.4, 50mM NaCl, 2mM EGTA, 2mM DTT, 0.02% Bry-35/NP-40 + 10% final glycerol and PI cocktail.

LB Medium (Luria-Bertani Medium): Per litre: 950 ml of deionized H_2O, 10 g tryptone, 5 g yeast extract, 10 g NaCl, pH 7.0. Autoclaved and stored at room temperature.

Lysis buffer / Sonication buffer (*E. coli*): 50mM Na_2HPO_4, 300mM NaCl pH 8.0.

Lysis Buffer (*Leishmania*): 10% glycerol, 50mM MOPS pH 7.2, 100mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100.

Lysis Buffer (Leishmania) for HA purification: 50 mM Tris, pH 7.5; 150 mM NaCl; 0.1% Nonidet P40.

Lysozyme: 5 $mgml^{-1}$ stock in 50mM Tris-HCl pH 7.4. Stored at -20°C.

Mounting Solution (For imunofluorescense slides): 50% glycerol, 2.5% DABCO, 1μgml^{-1} DAPI.

PBS: 10mM phosphate buffer, 2.7mM KCl, 137mM NaCl, pH 7.4. Autoclaved and stored at room temperature.

PCR mix (11.1x): 45mM Tris-HCl (pH 8.8), 11mM ammonium sulphate, 4.5mM $MgCl_2$, 6.7mM β-mercaptoethanol, 4.4mM EDTA pH 8, 1mM dATP, 1mM dCTP, 1mM dGTP, 1mM dTTP and 113 µg ml^{-1} BSA. Stored at -20°C.

RNAse A: 10mg ml^{-1} stock in sterile water. Stored at -20°C.

SDS-PAGE 4x Tris-Cl/SDS, pH 6.8: 0.5M Tris-Cl pH 6.8, 0.4% SDS.

SDS-PAGE 4x Tris-Cl/SDS, pH 8.8: 1.5M Tris-Cl pH 8.8, 0.4% SDS.

SDS-PAGE electrophoresis buffer:

SDS-PAGE sample loading buffer: (4x) 200mM Tris-HCl pH 6.8, 400µM β-mercaptoethanol, 8% SDS, 40% glycerol, a few crystals of bromophenol blue. Stored at room temperature. Alternatively, (6x) 300mM Tris-HCl pH 6.8, 8% SDS, 0.2% bromophenol blue, 60% glycerol, 600mM DTT. Stored at -20°C.

TE Buffer: 10mM Tris-HCl pH 7.4, 1mM EDTA pH 8. Stored at room temperature.

Western blot transfer buffer: 5mM Tris, 2mM Glycine, 20% methanol in distilled water.

Washing buffer for his tag purification: Sonication (Lysis) buffer + 50mM Imidazole. Alternatively, 20mM or 10mM Imidazole.

X-gal solution: Stock solution in dimethylformamide (dangerous!) at 20mgml^{-1}. Stored at 20^{o}C and away from light.

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